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CYTOTOXIC AND APOPTOTIC EFFECTS OF CAFFEIC ACID AND CAFFEOYL MALIC ACID ON CANCER CELLS

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in

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APPROVAL PAGE

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ABSTRACT

Urtica dioica, traditionally named stinging nettle is a species of the Urticaceae family. The leaf, flower, seed and root of *U. dioica* are used differently and contain different chemical components. Caffeic acid and caffeoyl malic acid are the phenolic compounds of *U. dioica*. In the present study, we aimed to determinetion the cytotoxic and apoptotic effects of different cocentrations (1, 5, 10, 50 μM) of Caffeic acid (CA) and Caffeoyl malic acid (CMA). We applied these chemicals on Glioblastoma (U87-MG), gastric adenocarcinoma (AGS), cervical carcinoma (HeLa), human embrionic kidney (HEK) lines by using xCelligence (real-time cell counter), TUNEL test (apoptotic test), Lactate dehydrogenease (LDH) and cell proliferation (WST-1) as a group of cytotoxicity tests. Our results show that CA especially in 50μM concentration has apoptotic and antiproliferative effects on HeLa, AGS, HEK and U87 cell lines at 48th hour. On the other hand CMA in 50 μM also has apoptotic and antiproliferative effects on HeLa, AGS, HEK and U87 cell lines. CMA may be used to treat certain cancer cell types alternatively.

Keywords: *Urtica Dioica*, Caffeic acid, Caffeoyl malic acid, HeLa, AGS, HEK, U87.

KAFEİK ASİT VE KAFEOİL MALİK ASİTİN KANSER HÜCRELERİ ÜZERİNE SİTOTOKSİK VE APOPTOTİK ETKİLERİ

Nurdan Sena NURDAĞ

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Tez Danışmanı: Doç. Dr. Lokman ALPSOY

ÖZ

Geleneksel ismi ısırgan otu olan *Urtica Dioica* Urticaceae ailesinin bir türüdür. *U. dioica* `nin yapraği, çiçeği, koku ve tohumu farklı kimyasallardan oluşmasıyla birlikte farklı alanlarda kullanılmaktadır. Kaffeik asit ve kafeoil malik asit *U.dioica* `nin fenolik bileşikleridir. Bu çalışma da bizim amacımız kafeik asit ve kafeoil malik asitin farklı konsantrasyonlarının gliablastoma (U87- MG), gastrik adenokarsinom (AGS), serviks karsinom (HeLa), insan embriyonik böbrek (HEK) hücre tipleri üzerine sitotoksik ve apoptotik etkilerinin belirlenmesidir. Sitotoksik ve apoptotik etkilerini belirleyebilmek için xCelligence (real-time cell counter), TUNEL test (apoptotik test), Laktat dehidrogenaz (LDH) ve hücre büyümesi (WST-1) yöntemleri kullanılmıştır. Bizim sonuçlarımız da kafeik asitin özellikle 50 µM`lik konsantrasyonun HeLa, AGS, HEK ve U87 hücre tipleri üzerinde 48. saatte apoptotik ve anti- proliferatif etkisinin olduğu görülmüştür. Öte yandan kafeoil malik asitin de aynı saatte 50 µM`lik konsantrasyonunun hücreler üzerinde apoptotik ve anti-proliferatif etkisinin olduğu görülmüştür. Kafeoil malik asit alternatif olarak farklı kanser hücre tipleri üzerine uygulanarak kullanılabilir.

Anahtar Kelimeler: Isırgan Otu, Kafeik asit, Kafeoil malik asit, HeLa, AGS, HEK, U87.

To my parents

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SYMBOL/ABBREVIATION	
AGS	Gastric Adenocarcinoma cell
CA	Caffeic Acid
CMA	Caffeoyl malic acid
CO_2	Carbon dioxide
DMEM	Dulbecco's modified Eagle's Medium
FBS	Fetal bovine serum
HEK	Human Embrionic Kidney cell
HeLa	Cervical Carcinoma cell
LDH	Lactate dehydrogenase
U87	Glioblastoma cell

CHAPTER 1

INTRODUCTION

1.1 *U. DIOICA* AND ITS COMPONENTS

Urtica dioica, traditionally named stinging nettle is a species of the Urticaceae. *U. dioica* is a perennial herb with rhizomes, stolons and abundant stinging trichomes. The plant is spreading various region of the world. It prefers nutrient riches and lighted places. It is grown specially in Black sea region and other parts of Turkey. Also, it is grown in North and South Africa, China, India, Australia, New Zealandand North and South America and native throughout Europe (Otles 2012). The roots, seeds and leaves of *U. dioica* have been used in folk medicine in the world. It has benefical effects on health such as nasal and menstrual haemorrhage, diabetes, rheumatism, eczema, anaemia hair loss, hypotensive, anti-inflammatory effects, prostatic hyperplasia, diuretic and immunomodulatory activity. In addition some extracts or components of *U.dioica* have been using for cancer patients. The leaves, seeds and roots of *U. dioica* contain different chemical compounds. It consists of glucopyranosides, glycoprotein, protein, flavonol glycosides, carotenoids as well as biologically active compounds, such as caffeic acid, caffeoyl malic acid and caffeoyl quinic acid, essential oils, formic and acetic acid, histamine, tannins, mucilage, vitamins (A, B1, B2, C, K1, folic and pantothesic acids (A.H. Mahmoud 2005). The one of the contents of *U. dioica*, phenolic compounds are large and diverse group of molecules, which includes many different families of aromatic secondary metabolites in plants (Nicolas Rispail 2005). One of the most important groups of these metabolites are phenolic compounds are caffeic acid and caffeoyl malic acid (Michalak 2006). They have biological protective effects on human health such as against oxidative damage, coronary heart disease and high blood pressure, diabetes, cancer, inflammative, viral and parasitic disease, psychotic disorders

(Prasad, Jeyanthimala et al. 2009). We give our attention to the two of the phenolic compounds in *U. dioica* in this thesis. These are caffeic acid and caffeoyl malic acid. Caffeic acid one of the most common phenolic acids, frequently occuring in fruits, vegetables, grains, dietary supplements and traditional herbs like *U. dioica* (Qiang 2011). It has a lot of pharmacological effects on diseases such as antioxidative activities, enzyme activity inhibition, antitumor activity, anti-inflammatory properties and inhibition of HIV replication. And also caffeic acid to be an inducer of apoptosis in cancer cell lines and capable of tumor growth inhibition (Bhat, Azmi et al. 2007). Although there are a lot of studies about the effects of caffeic acid on cancer cell lines, there are no any studies on the effects of caffeoyl malic acid on cancer cell lines, with previous work demonstrating the presence of caffeic acid and caffeoyl malic acid. That's why we thought that both caffeoyl malic acid and caffeic acid are components of Urtica dioica and they have similar structure so caffeoyl malic acid may be able to show the same effect as caffeic acid does. The aim of this study is to determinetion the cytotoxic and apoptotic effects of caffeic acid and caffeoyl malic acid at different concentrations on cancer and healthy cell lines in vitro.

1.2 SCIENTIFIC CLASSIFICATION OF *URTICA DIOICA*

Family Urticaceae (nettle family) is a part of the larger group Order Urticales and mostly tropical and subtropical in both hemispheres. Mabberley (1997) lists 48 genera with 1050 species for Urticaceae (see Table 1) (Coile 1999). Popular name is stinging nettle and it belongs to the family of Urticaceaea (Aksu and Kaya 2003). The main varieties are designated below the Urtica species are *Urtica dioica* L., *Urtica urens* L., *Urtica pilulifera* L., *Urtica cannabina* L., *Urtica membranacea* Poiret, *Urtica kiovensis* Rogoff (Kavalalı, Tuncel et al. 2002).

Table 1. Scientific classification of Urtica dioica.

Kingdom	Plantae- plants
Phylum	Magnoliophyta-Flowering plants
Class	Rosopsida
Order	Rosales
Family	Urticaceae
Genus	Urtica
Species	*U. Dioica*

1.3 DESCRIPTION OF *U. DIOICA*

Urtica dioica is a perennial herb with a spacious sympodial system of rhizomes and stolons, rooting at the nodes and that rise in leap to aerial shoots up to 1.5–2 m or (rarely) 3 m, or more, stems and leaves have with abundant remarkable stinging hairs (Kavalalı, Tuncel et al. 2002; Taylor 2009). These are distinguished with stinging hairs. Leaves are opposite, flowers are green with yellow stamens (see Figure 1.1 a) and the male (see Figure 1.1 b) and female (see Figure 1.1 c) flowers on separate plants. The fruits of plants are achene (see Figure 1.1 d). These are the characters of Urtica genus (Kavalalı, Tuncel et al. 2002). However *U. dioica* is a dioecious plant with opposite, sharply toothed leaves and stinging trichomes (see Figure 1.2 e) growing to a height of 30–100 cm (see Figure 1.2 f). It is nitrophilous herb containing sclerenchymatic fibers in the bark (Patrizia Pinelli and Silvia Baronti 2008, Oguz 2012). *U. dioica* is a wind pollinated, herbaceous, rhizomatous perennial. It occurs in alluvial woods, margins of deciduous forests, fence rows and waste places (Shannon 2005).

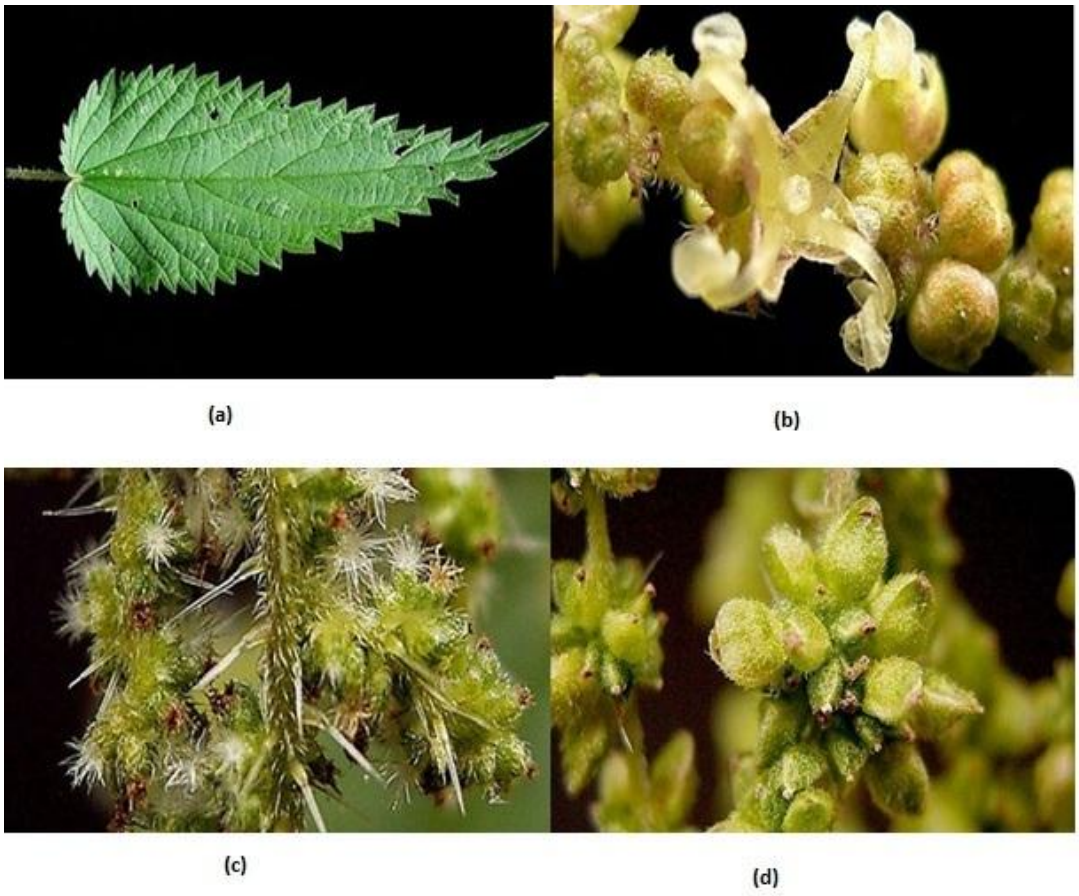

Figure 1.1 Leaf (a), male flower (b), female flower (c) and fruit (d) of Urtica dioica.

Figure 1.2 Stinging trichomes (e) and the structure (f) of Urtica dioica.

1.4 DISTRIBITION OF *U. DIOICA*

U. dioica naturally is founded in pathway, field, and wildwood. They are grown in bottom of barriers, ruins and hot and mild climate areas, grassy places, between cultivated plants, street, and water runnels. This plant prefers nutrient riches and lighted places. They are exhibited wide distribution of the world. In Turkey it is also grown in the different regions. Some local names of nettle are “dalagan, dizlagan, agdalak, and isirgan”. This plant which is specially grown in Black Sea region has reach chemical composition (Otles 2012). In the world *U. dioica* is extensived and probably native throughout Europe (see Figure 1.3) and Asia from the arctic regions to the Mediterranean. In addition worldwide *U. dioica* is occured in other temperate regions in North and South Africa, China, India, Australia, New Zealandand North and South America, but not found in the tropics (see Figure 1.4) (Taylor 2009).

Figure 1.3 The distribution of the U. dioica group in Europen (Taylor 2009).

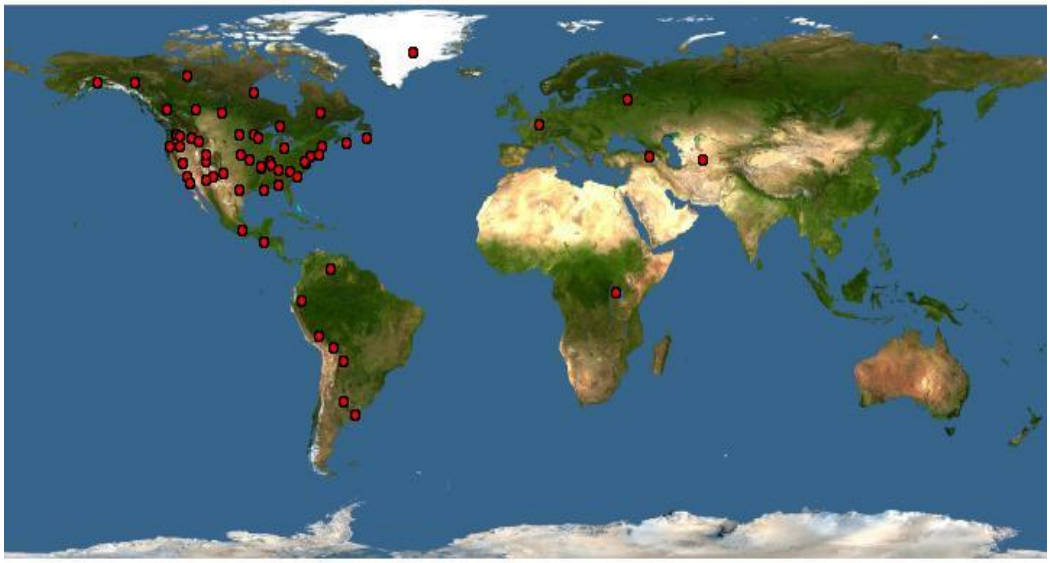

Figure 1.4 This map that shows where, throughout the world, stinging nettles have been found.

1.5 USES AREAS AND EFFECTS OF *URTICA DIOICA*

Urtica dioica is used such as a drug, food, fibrous, dye, and cosmetic and medical and pharmacologic researches about nettle are increased day by day. It has a long history such a both herbal cure and nutritious addition to the diet which is easily digested. The plants are used principally in soup, a tea made from the leaves has been used and traditionally employed such a folk medicine for a wide spectrum of diseases. *U. dioica* for intake have beneficial effects in health (Guil-Guerrero, Rebolloso-Fuentes et al. 2003, Ozen and Korkmaz 2003). *Urtica dioica* todays, it is contained into a number of herbal medicinal preparations (Ozen and Korkmaz 2003). The leaves and roots are used as a blood purifier and diuretic, an infusion of the plant is used for nasal and menstrual haemorrhage, against diabetes in folk, rheumatism, eczema, anaemia hair loss, to show hypotensive, anti-inflammatory effects, to be useful in the therapy of prostatic hyperplasia, to show diuretic, immunomodulatory activity as an expectorant infections. Thus the root has been shown to have a beneficial effect upon enlarged prostate glands (Kavalalı, Tuncel et al. 2002, Akbay 2003, Guil-Guerrero, Rebolloso-Fuentes et al. 2003). In Europe the aqueous extract from roots of *U. dioica* has been succeed to used in clinics for the treatment of prostatic hyperplasia. In Turkey, the seeds, leaves, as well as flowers and roots and the aqueous extract of the aerial parts of

U. dioica are generally used such a herbal medicine by cancer patients and are used to treat stomacache and against liver insufficiency in Turkish folk medicine. Also some reports of positive results are existed (Akbay 2003, Ozen and Korkmaz 2003, A.H. Mahmoud 2005). In addition, *U. dioica* demonstrate antioxidant activity against iron-promoted oxidation of phospholipids, linoleic acid, and deoxyribose and prevent the damage of rat liver tissue structure (A.H. Mahmoud 2005 and Gulcin, Kufrevioglu et al. 2003) That has been also discovered the leaf extracts from *U. dioica* have an anti-inflammatory effect owing to its inhibitory effect on NF-6B activation, a family of transcription factors which are critical for the inducible expression of many genes involved in inflammatory responses (Guil-Guerrero, Rebolloso-Fuentes et al. 2003).

1.6 THE CONTENTS OF *URTICA DIOICA*

U. dioica is comprised of various different components. It includes glucopyranosides, glycoprotein, protein, flavonol glycosides, carotenoids as well as biologically active compounds, such as caffeoyl malic acid and caffeoyl quinic acid, essential oils, formic and acetic acid, histamine, tannins, mucilage, vitamins (A, B1, B2, C, K1, folic and pantothesic acids (A.H. Mahmoud 2005). Main constituents identified in the plant material are summarized in Table 1.2 (Chrubasik, Roufogalis et al. 2007). These compounds as steroids, terpenoids, phenylpropanoids, lignans, coumarins, polysaccharides and lectins (*Urtica dioica* agglutinin) have been isolated from the roots of the plant (Akbay 2003). Caffeoyl malic acid and caffeic acid are phenolic compound of *U. dioica* were investigated in this study.

Table 1.2 Main constituents identified in nettle herb (Chrubasik, Roufogalis et al. 2007).

Flavonoids
Glucosides and rutinosides of quercetin, kaempferol and
isorhamnetin
Caffeoyl-esters
Caffeoylmalic acid (only Urtica dioica)
Chlorogenic acid
Neochlorogenic acid
Caffeic acid
Scopoletin (Cumarin)
Sitosterol (-3-O-glucoside)
Polysaccharides
Fatty acids (e.g. 13-hydroxyoctadecatrienoic acid)
Minerals (Herba: up to 20%; leaves: 1–5%)

1.6.1 Phenolic Compounds

Phenolic compounds are obtained from secondary metabolites in plants, are natural phytochemicals from phenylalanine, less often from tyrosine, and are widely present in food and nutraceuticals. Chemically, phenolic compounds are diverse according to the number of hydroxylated aromatic rings and the type of functional moiety, they can be simply categorized such as phenolic acids, polyphenols, or monophenols. Phenolic compounds are defined as biologically active and herbal and have positive effects on health. They have protective effects against oxidative damage, coronary heart disease and high blood pressure, diabetes, cancer, inflammative, viral and parasitic disease, psychotic disorders. Recently, evidence suggesting phenolic compounds possess an effective inhibitory effect on cancer invasion and metastasis is also increasingly being reported in the scientific literature Otles (2012) and Weng and Yen (2012). In addition to flavonoids and other polyphenolic compounds derived from fruits and berries have been shown to induce apoptotic pathways and to suppress proliferation of various types of cancer cells such as leukemic cells and liver cancer cells (Chang, Hsieh et al. 2010). For instance polyphenols can be acted as suppressing agents, and inhibited the formation and growth of tumors from initiated cells because they inhibit cell proliferation *in vitro*. (Rocha-Guzmán, Gallegos-Infante et al. 2009 and Weng and Yen 2012).

Previous studies demonstrated that caffeic acid exhibits an antiproliferative effect against cervix (HeLa), mammary gland adenocarcinomas (MDA-MB-231), lymphoblastic leukemia (MOLT-3), and non-neoplasic fibroblasts from human embryonic lung tissue (L-132 cell line) (M. Touaibia 2011) and also induces apoptosis of lung carcinoma (A549), non-small lung carcinoma (H1299). This antiproliferative effect, in which NF-κB is involved in the protective effect by caffeic acid, reduces the cell growth of leukemic monocyte and also inhibits angiogenesis of renal carcinoma cancer cell lines (Lin, Chen et al. 2012). Moreover a previously demonstration which also revealed inhibitory activity of caffeic acid against colon cancer cell (HT 29, HCT 15) and hepatocellular carcinoma (HepG2) in which cell proliferation was in a dose-dependent manner. It was shown that caffeic acid is blocked the MMP-9 expression by inhibiting the NF-κB activity (Jaganathan 2012). In addition caffeic acid shows an antiproliferative effect particularly towards HeLa (M. Touaibia 2011). On former researches different concentrations of caffeic acid has been studied to examine the effects of caffeic acid on proliferation of diverse cells. To begin with, non small cell lung carsinoma NSCLC cells for which 50 and 100 μM concentrations performed. Actually in t assay caffeic acid exerts no significant cytotoxicity at the dose 50 and 100 μM towards NSCLC A549 cells. The doses (0, 50, 100, and 150 μM) of caffeic acid were applied on cells for 48 h (Lin, Chen et al. 2012). Secondly, HeLa cells were then incubated with different concentrations of caffeic acid (0.5, 1, 2.5, 5 or 10mM) for 24 hours. To examine the effects of caffeic acid on HeLa cells, cells are treated with various concentrations of caffeic acid (Chang, Hsieh et al. 2010). A recent work showed that caffeic acid at a dose of 20 mg/kg retarded the growth of HepG2 tumor xenografts in immunosuppressive mice. 2500 μM of caffeic acid was found to inhibit 50% cell proliferation of HT 29 cells (Jaganathan 2012). Last but not least, the effect of caffeic acid on metastasis formation was studied in mice injected in vitro with tumor cells. Tested caffeic acid was given at the dose of 50 mg/kg or 150 mg/kg for caffeic acid (Orsolic, Knezevic et al. 2004).

1.6.1.1 Properties and Different Effects of Caffeic Acid

A major class of polyphenolic compounds in plants is hydroxycinnamic acids. Hydroxycinnamic acid derivatives are a major category of phenolic acids that may be

provided important effects such as preventing cancer, atherosclerosis, heart disease etc. (Maurya and Devasagayam 2010). It is a widespread phenolic acid naturally occurring polyphenol widely in many agricultural products such as fruit, vegetables, wine, tea, apple juice, olive oil, and coffee, bean and therefore significantly present in human diet (Mirella Nardini 2001). Hydroxycinnamic acids like caffeic, ferulic, sinapic and p-coumaric acids are present in a large variety of fruits and vegetables including blueberries, grapes, apples, cereal brans, broccoli, spinach and lettuce. The major agent of these compounds is caffeic acid (Bhat, Azmi et al. 2007, Marie-Paule Gonthier 2011). Caffeic acid is a known plant phenolic acid, and the phenolic acids in plants are known as phytochemicals (Ye, Hsiao et al. 2010). Vegetables sources of caffeic acid include apples, pears, berries, artichoke and aubergines (Prasad, Jeyanthimala et al. 2009). The molecular structure of caffeic acid (see Figure 1.5) containing a catechol group with an a,b,-unsaturated carboxylic acid chain is responsible for its efficient interaction with several types of oxidant radicals (Medina, Undeland et al. 2012).

It is absorbed in humans after oral administration and specific metabolites are detected in the urine. Caffeic acid is transformed by the intestinal microflora of man and experimental animals. Urinary metabolites that appear after the ingestion of caffeic acid indicate that these transformations are also the basis of caffeic acid metabolism in man and experimental animals (Goldman 1972). However caffeic acid has a wide spectrum of biological and pharmacological effects, including antioxidative activities, enzyme activity inhibition (5- and 12- lipoxygenases, glutathione S-transferase, xanthine oxidase), antitumor activity, anti-inflammatory properties, inhibition of HIV replication and the absorption of cholesterol in the body, reducing the levels of cholesterol, phospholipids, free fatty acids and triglycerides (Mirella Nardini 2001) (Ye, Hsiao et al. 2010, Medina, Undeland et al. 2012).

In addition, it has been reported that caffeic acid acts as an antitumor promoter on forestomach, liver, skin carcinogenesis, oral cancer cell growth and suppress colon carcinogenesis. It can also be a factor in the formation of lymphocytes in the immunity process (Akihiro Hagiwara 1991, Ye, Hsiao et al. 2010). The ability of caffeic acid inhibit the activity of several protein kinases such as phosphorylase kinase, protein kinase C (PKC), and protein kinase A (PKA) suggests a more direct and specific

involvement of this molecule in the modulation of cellular functions not necessarily associated with its antioxidant activity (Mirella Nardini 2001).

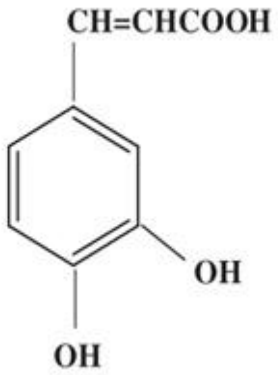

Figure 1.5 The molecular structure of caffeic acid.

1.6.2.2 Description of Caffeoyl Malic Acid

Caffeoylmalic acid is one of the active component in U. dioica (see Figure 1. 6). The quantity of caffeoylmalic acid in Urtica is not only on the origin of the plant and highest concentrations are found in the leaves (Bauer R 1997). Previous studies demonstrated that a nettle leaf hydro-ethanolic extract and its main phenolic constituent caffeoyl malic acid were tested for their inhibitory potential on biosynthesis of arachidonic acid metabolites by rat leukaemic basophilic granulocytes (RBL-1 cells). It has been showed that Extractum Urticae dioicae and the main phenolic ingredient of caffeoylmalic acid inhibit the biosynthesis of cyclooxygenase derived reactions (IC50 extract IDS-23 92 μg/mL, caffeoyl malic acid 38 μg/mL) and caffeoylmalic acid inhibited the leukotriene B4 synthesis in a dose-dependent manner. Both the extract and the caffeoyl malic acid also have been showed a strong concentration-dependent inhibition of synthesis of cyclooxygenese-derived prostaglandins [IC50 of 92 μg/mL for extract (PGD2) and 38 μg/mL for the acid (PGD2 and PGF2α)] (Obertreis B 1996).

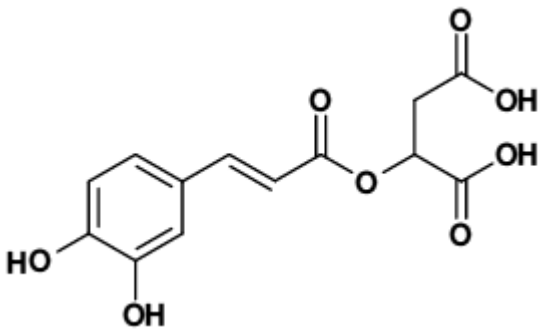

Figure 1.6 The structure of Caffeoyl malic acid.

1.7 LDH ASSAY

The Roche LDH Cytotoxicity Detection Kit is designed to be a simple method to directly quantify cell death in culture. Cell death can be assayed by quantifying plasma membrane damage or rupture. This LDH assay kit offers a simple way to measure plasma membrane damage, based on the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in most cells. The LDH Cytotoxicity Detection Kit is a simple and accurate colorimetric assay for dead and damaged cells. LDH present in the culture supernatant (due to plasma membrane damage) participates in a coupled reaction which converts a yellow tetrazolium salt into a red, formazan-class dye which is measured by absorbance at 492 nm (see Figure 1.7). The amount of formazan is directly proportional to the amount of LDH in the culture, which is in turn directly proportional to the number of dead or damaged cells. The assay is extremely sensitive: as few as 2,000 dead or damaged cells per well can be detected.

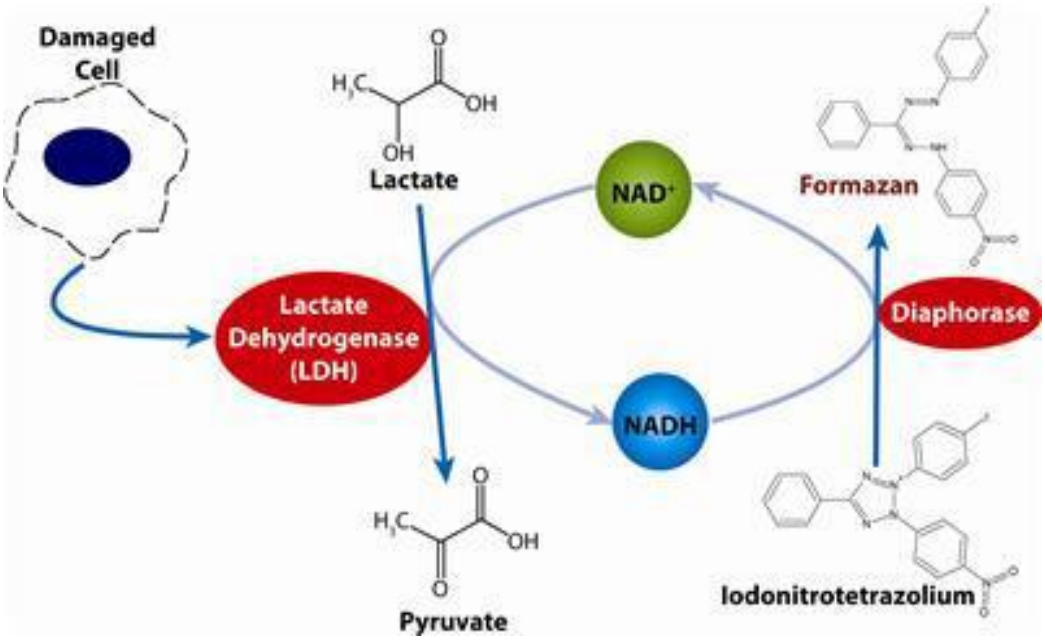

Figure 1.7 LDH assay Principe. In the first step, released lactate dehydrogenase (LDH) reduces NAD+ to NADH+ H+ by oxidation of lactate to pyruvate. In the second enzymatic reaction 2 H are transferred from NADH+ H+ to the yellow tetrazolium salt INT by a catalyst.

LDH assays have been used to quantify cell-mediated cytotoxicity induced by cytotoxic T cells, natural killer cells, lymphokine-activated killer cells, and monocytes, as well as to identify mediators that induce cytolysis. LDH assays have also been used to determine the cytotoxic potential of compounds in environmental and medical research and in food, cosmetic, and pharmaceutical manufacturing, and to detect cell death in bioreactors. Additionally, the assay is nonradioactive for improved safety and simplified cleanup and waste disposal.

1.8 WST-1 ASSAY

A water-soluble tetrazolium salt (WST-1) assay was developed to quantify cell proliferation and viability for a wide range of pharmacological and functional applications. That is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases to form formazan in viable cells (see Figure 1.8). The greater the number of viable, metabolically active cells, the greater the amount of formazan product

produced following the addition of WST-1. By detection of the formazan level in the cells, we can quantify the cell number. The assay can be used not only for quantifying cell proliferation, but cytotoxicity can also be measured. The solubility of reduced WST-1 salt makes the WST-1 assay the most convenient and commonly used tetrazolium salt technique despite the fact that its reading scan be significantly affected by cell genotype and experimental conditions (Weir, Robertson et al. 2011). Quantification of the formazan dye produced by metabolically active cells by a scanning multiwall spectrophotometer (ELISA reader). The absorbance of the dye solution is measured at appropriate wavelengths.

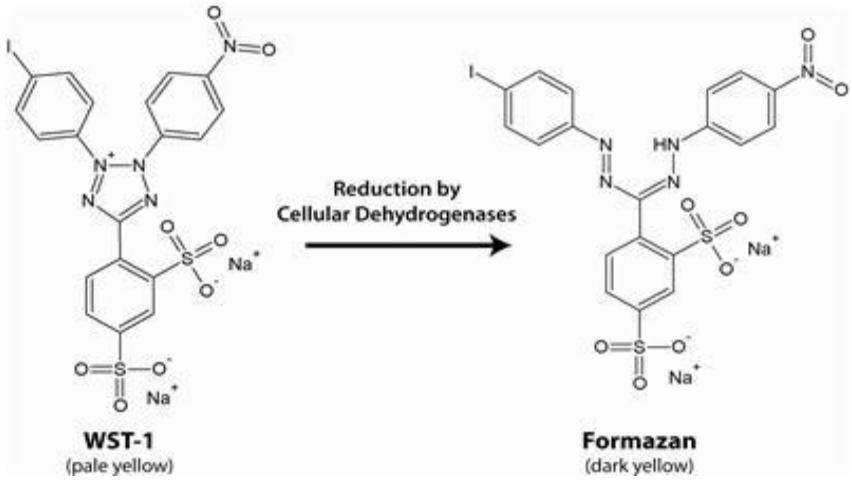

Figure 1.8 The assay principle is based upon the reduction of the tetrazolium salt WST-1 to formazan by cellular dehydrogenases. The generation of the dark yellow colored formazan is measured at 420-480nm (optimal at 440nm) and is directly correlated to cell number.

1.9 Xcelligence SYSTEM

The Xcelligence System is a labeling free cell–based assay system integrating micro electronics and cell biology, suitable for uninterrupted monitoring of biolgical processes of living cells (Zhang 2012). The impedance measurement provides quantitative information about the biological status of the cells, including cell number, viability, and morphology. The Xcelligence system consists of the RTCA analyzer, the RTCA SP station, the RTCA computer with integrated software, and disposable E-plate

96 (see Figure 1.9) (Urcan, Haertel et al. 2010). The System measures electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates. Cells that have contact with the sensor change the electrical impedance between the microelectrodes. For example while there are no cells on a microelectrode surface, the system's electronic lineament will not be effected and the impedance change will be zero. When attaching of one cell onto the electrodes, impedance will be 1. When more cells attach onto the electrodes, the impedance will be increased. Xcelligence system allows for the calculation of optimized EC50 values in realtime (see Figure 1.10). Although cell proliferation leads to a higher CI value for adherent or spread cells, cell death or toxicity induces cell-detachment so this is lead to a decreased CI value (Urcan, Haertel et al. 2010). The wide variety of applications include; compound-mediated and cell-mediated cytotoxicity, cell adhesion and cell spreading, cell proliferation and differentiation, receptor-mediated signaling, virus-mediated cytopathogenicity, constant quality control of cells, cell invasion and migration assays.

Figure 1.9 Xcelligence RTCA Instruments.

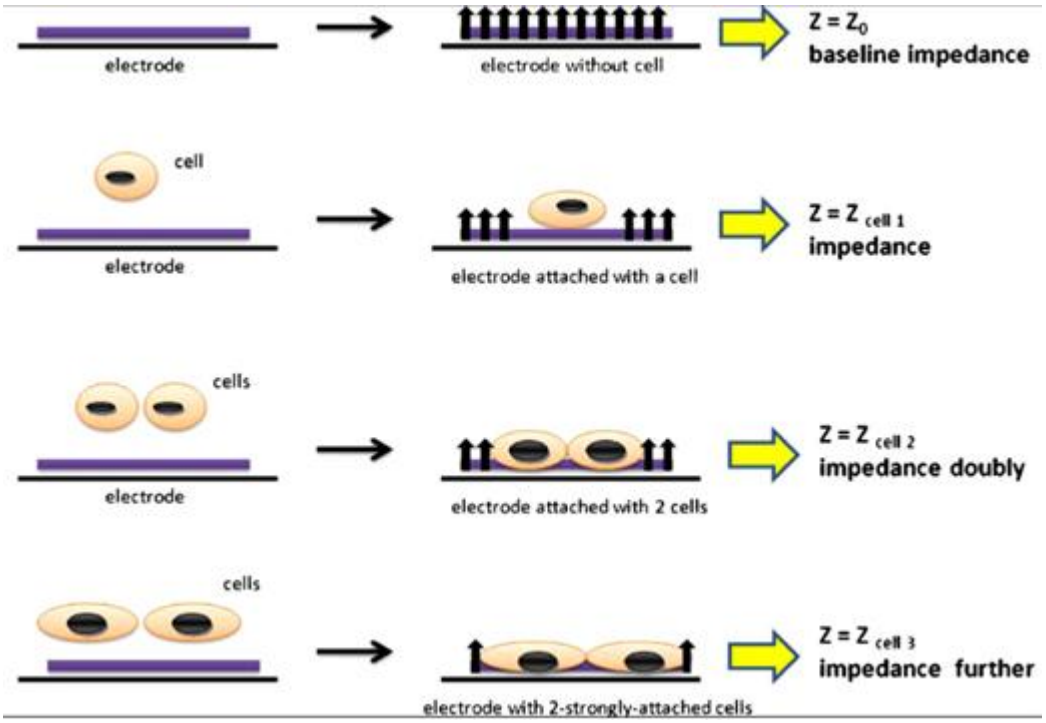

Figure 1.10 Scheme of impedance measurement. (Urcan, Haertel et al. 2010).

1.10 TUNEL ASSAY

Apoptosis, also known as programmed cell death, is a normal process of the development and health of multicellular organisms. When a cell's DNA becomes damaged, a cell can undergo apoptosis, preserving the healthy state of the organism. It is also beneficial as a natural anti-cancer mechanism. Apoptosis functions in multiple contexts. The one of change include DNA fragmentation have occurred during apoptosis. And also during apoptosis, DNAse activity not only generates double-stranded, low-molecular-weight DNA fragments (mono-and oligonucleosomes), but also introduces strand breaks ("nicks") into the high-molecular-weight DNA (see Figure 1.11 a). These processes can be identified by labeling the free 3'-OH termini with terminal transferase (TdT), which attaches labeled nucleotides to all 3´OH-ends (TUNEL reaction; TdT-mediated dUTP nick end labeling) (see Figure 1.11 b). These methods, known as TUNEL, can effectively detect late apoptosic cells. You can use flow cytometry, light microscopy, fluorescence microscopy and/or microplate assays. This labeling is more sensitive than other methods, and is the method used by the Roche Applied Science In situ Cell Death Detection Kits.

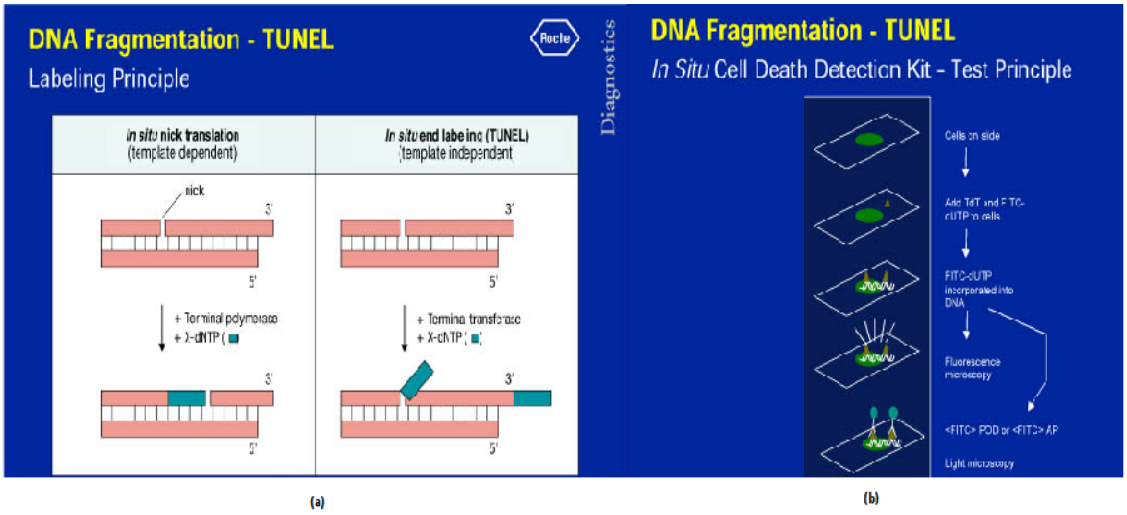

Figure 1.11 DNA fragmentation (a) and TUNEL Labeling Principle (b).

CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 General Chemical Reagents

All of the general laboratory chemical reagents were given as a list in Table 2.1 in these works. We used caffeic acid and caffeoyl malic acid which are chemicals for this work.

Table 2.1 List of general chemical reagents and their brands.

Chemical Reagents	Brands
PBS	Biochrome AG
FBS	Biochrome AG
Trypsin	Biochrome AG
DMEM	Gibco
Prymocin	Biological Industries
4 % paraformaldehyde	(4 g paraformaldehyde- 100 ml PBS)
DMSO (Dimethyl sulphoxide)	Merck
Caffeic acid	Biological Industries
Caffeoyl malic acid	Biological Industries
Mitomycin C	Sigma
Triton X 0,1%	Roche
In Situ Cell Death Detection Kit	Roche (Germany)
WST-1 Kit	Roche (Germany)
Cytotoxicity Detection Kit (LDH)	Roche (Germany)

2.1.2 Equipments

All of the general laboratory equipment were given as a list in Table 2.3 in these works.

Table 2.1 List of the general equipment and their brands.

Equipments	Brand and Model
Centrifuge	Hettich , Mikro 22
Vortex	Vortex IKALABOTECHNIK
Water Bath	Water Bath Nuve
Inverted Light Microscope	Inverted Light Microscope Zeiss
CO^2 Incubator	CO^2 Incubator Thermo ,Sanko
Microfilter (1.0/0.45 µm) GF/PET	Microfilter (1.0/0.45 µm) GF/PET
Pipettor	Reddot Hirschmann Laborgerate
Centrifuge Tubes Falcon	Micropipettes Nichiryo
Trypan blue	Sigma
Water purification system	Millipore
Laminar Flow Hood	Esco , Kotterman
Equipments	Brand and Model
Serological Sterile, Plastic pipets	(2 mL, 5 mL, 10 mL)
Autoclave	CERTO CLAW A-4050 Traun, Austria
Water Purification System	Millipore
Flask	(25 cm^2, 75 ^{2} , 150 cm^2)
96-well plates	(25 cm^2, 75 ^{2} , 150 cm^2)
ELISA reader	Biotek

2.2 METHODS

2.2.1 Cell Culture

Glioblastoma cell (U87- MG), gastric adenocarcinoma (AGS), cervical carcinoma (HeLa), human embrionic kidney (HEK) cell lines were chosen for observing effects of caffeic acid and caffeoyl malic acid *in vitro.*

2.2.2 Thawing of Cell Lines (AGS, HeLa, HEK, U87)

Previously 10 ml of warmed up Dulbecco's Modified Eagle's Medium (DMEM-LG, Gibco) at 37ºC was transferred to the 15 ml falcon tubes. Then cryovial tubes were taken from the liquid nitrogen tank (-196ºC). They were transferred into the 37ºC water bath and shaken slowly until melting. The contents in the cryovial were transferred to the 15 ml falcon tube bearing DMEM as quickly as possible and centrifuged at 2000 rpm for 3 min. The supernatant was discarded to get rid of DMSO (Dimethyl Sulfoxide, Applichem) and other unwanted cellular products from the medium. The remaining part is pellet was resuspended by finger mixing. Then living cells were counted. After that cells were seeded into a 25 cm^2 tissue culture flask (Greiner) in 10 ml DMEM including 20% Fetal Bovine Serum (FBS, Biochrom) and 1% Penicillin/Streptomycin (Biochrom). Cells were incubated in a humidified atmosphere (37°C, 5% CO_2) and the screw cap of the flask was kept flexible to allow circulation of CO_2 into flask. The day after medium was refreshed to allow eliminate the dead cells.

2.2.3 Subculture of Cells Lines

After seeding of cells, the cells in the flask, the cells were attached and confluenced on the surface of the flask. When the cells get %80-90 of confluesy, subculture is done. Before DMEM, FBS, Phosphate Buffered Saline (PBS, Biochrom) and trypsin were heated up to 37^oC at water bath. Medium was removed by a sterile pipette and cells were washed with 5 ml of PBS in the flask. Then PBS was removed, cells were trypsinized with pre-warmed 4 ml of 0.2 % Trypsin/EDTA solution (Gibco) and cells were incubated in humidified atmosphere (37°C, 5% CO_2) for 5-10 min. after that the cells were observed under the inverted microscope. When the cells detached on the surface of flask, FBS (1.5 ml) was added in the flask to neutralize effect of Trypsin/EDTA solution. The neutralized cells in the flask with trypsin and FBS were transferred into a 15 ml centrifuge tube and then centrifuged at 2000 rpm for 3 min at room temperature. After centrifugation, supernatant was discarded until 0.5 ml of the cell suspension at the bottom of the tube. Pellet was finger mixed and the tube was completed 10 ml with DMEM medium in order to washing cells. Centrifuge was repeated. Then cells were counted with hemocytometer. After counting, cells were seeded at a density of 1×10^4 cells/cm^2 with DMEM containing 10% FBS for expansion.

Subculture of cells was repeated at about 3-4 days intervals according to the cell types and doubling times.

2.2.4 Freezing of Cell Lines

For the freezing process, cells were re-suspended in FBS at density of 1-2x106 cells/ml. Cryovial tubes were placed on ice and 950µl cell suspension was transferred into each tube. 50 µl DMSO (Dimethyl Sulfoxide, Applichem) was added into each tube drop by drop and cell suspension was mixed by pipetting. Tubes were left at -20^{0}C for 1 h and then kept at -80^{0}C overnight. Then cryovial tubes were transferred to the liquid nitrogen tank (-196 ^{0}C) to protection for a long time.

2.2.5 Adding Caffeic acid and Caffeoyl malic acid

Caffeic acid and caffeoyl malic acid were prepared at different concentration with serial dilutions to add the cell lines (see Table 2.4 and Table 2.5). This proportion estimated with Xcelligence system and proliferation tests.

Table 2.2 The concentration of caffeic acid (CA).

Groups	Concentrations
CA stock solution	400 µM
CA 1	50 µM
CA 2	10 µM
CA 3	5 µM
CA 4	1 µM

Table 2.3 The concentration of caffeoyl malic acid (CMA).

Groups	Concentration
CMA stock solution	400 µM
CMA 1	50 µM
CMA 2	10 µM
CMA 3	5 µM
CMA 4	1 µM

2.2.6 Cytotoxicity Assay

AGS, HEK, HeLa, U87 cell lines were seeded in triplicate at $1x10^4$ cells/well to the 96 well plates in 100 µl medium and then pre-incubated for 24 h before the treatment. Following exposure to the determinant concentrations of CA and CMA, cytotoxicity for cell lines at 24 and 48h were analyzed with the lactate dehydrogenase (LDH) leakage assay (Roche). The assay conducted immediately by mixing the media with the assay reagent which was prepared by mixing two separate solutions. This was incubated for 30 minutes, protected from light, and the absorbance was then read at 490 nm, with a reference reading at a wavelength above 600 nm.

2.2.7 Proliferation Assay

AGS, HEK, HeLa, U87 cell lines were seeded in triplicate at 1x104 cells/well to the 96 well plates in final volume of 100 µl/well culture medium in a humidified atmosphere (37°C, 5% CO_2) for 24 h before the treatment. After that cell lines were exposured to the determined concentrations of both CA (1, 5, 10, 50 µM) and CMA(1, 5, 10, 50 µM). Cell proliferation was evaluated for AGS, HeLa, HEK and U87 cell lines at 24 and 48 h were determined with the WST-1 cell proliferation assay (Roche). Cell Proliferation Reagent WST-1 was added in a 10 µl/well volume. Cells were incubated for 4h in a humidified atmosphere (37°C, 5% CO_2). The absorbance of samples were measured at 420 nm with ELISA reader.

2.2.8 Xcelligence System For Analyzing The Cell Proliferation

AGS, HeLa, HEK and U87 cell lines were grown and expanded in tissue-culture flasks. After reaching 75% confluence, the cell lines were washed with PBS, afterwards detached from the flasks by a short time treatment with trypsin/EDTA. Subsequently, 100 µL of cell culture media at room temperature was added into each well of E-plate 96. After this the E-plate 96 was connected to the system and checked in the cell culture incubator for proper electrical-contacts and the background impedance was measured during 24s. The cell lines were resuspended in cell culture medium and adjusted to 10.000cells/mL at the same time. 100 µL of each cell suspension was added to the 50 µL medium containing wells on E-plate 96, in order to determine the optimum cell concentration. After 30min incubation at room temperature, E-plate 96 was placed into the cell culture incubator. Finally, adhesion, growth and proliferation of the cells were monitored every 15min for a period of up to 24h via the incorporated sensor electrode arrays of the E-Plate 96. The electrical impedance was measured by the RTCA-integrated software of the Xcelligence system as a dimension less parameter termed CI. The proliferation, attachment and spreading of the cells were monitored every 15min by the Xcelligence system. Approximately 24h after seeding the medium in the each well was discarded in order to get rid of the dead cells. When the cells were in the log growth phase, the cells were exposed to 100 µL refresh DMEM containing the following substances: CA (1, 5, 10, 50µM) and CMA (1, 5, 10, 50 µM). DMEM and 10% FBS were used for control. All experiments were run for 48h. Cell index (CI) is derived to represent cell status based on the measured relative change in electrical impedance that occurs in the presence and absence of cells in the wells Impedance is measured at 3 different frequencies (10, 25 or 50kHz) and a specific time.

2.2.9 TUNEL Assay

DNA fragmentation is an indicator for detecting the late apoptosis. TUNEL (Roche) is an assay that is composed of an enzyme solution and labeling solution which detects the nicks (single strand breaks) in DNA and binds to free 3'-OH ends. Adding of dUTPs to 3'-OH ends by terminal deoxynucleotidyl transferase causes labeling of DNA. Fluorescently labeled ends were detected by using confocal microscopy (Leica). AGS, HeLa, HEK and U87 cell lines were used for this experiment. The cell lines were grown for TUNEL assay. AGS, HEK, HeLa, U87 cell lines were seeded at $1x10^4$ cells/well to

the 96 well plates in final volume of 100 µL/well culture medium in a humidified atmosphere (37°C, 5%CO_2) for 24 h before the treatment. Then the next day the highest concentration of CA and CMA which was 50 µM concentration was chosen for all of the cell lines. The cells were treated with 50 µM CA and CMA for 48 h. Mitomycin C was used as the positive control. After the incubation period, the cells were washed twice with phosphate buffer solution (PBS), then fixed with 4 % paraformaldehyde for 60 minutes and washed again twice with PBST (PBS+0,1% Triton-X-100) on the shaker. 0,1% Triton X were treated with 200 µL with cells on the ice for 10 minutes. After that the cells were washed twice with PBST on shaker. TUNEL reaction mixture was added 50 µl and incubated at 37°C in a humidified atmosphere with 0.5% CO_2 for 1h . After that the cells washed with PBST. And then DAPI was applied on the cells for 15 minutes then washed once PBST and once distilled H_2O. The results were analyzed by fluorescence microscopy.

2.2.10 Statistical Analysis

For the statistical analysis of WST-1 and LDH values, Mann– Whitney U-test was used. Also for TUNEL assay values X^2 test was used. Statistical differences between time, and dose were analyzed. A value of P less than 0.05 was accepted as statistically significant. Results were expressed as mean ± SE. For these procedures, SPSS 11.5 version for Windows (SPSS Inc, Chicago, Illinois, USA) was used.

CHAPTER 3

RESULTS

3.1 THE EFFECTS OF CAFFEIC ACID ON HELA CELLS

At 24^{th} and 48^{th} hours in LDH assay, there was no effect of caffeic acid on HeLa cells. This result may show caffeic acid has no effect on lipid peroxidation, either. On the other hand, according to WST-1 results, caffeic acid decreased cell number at both 24^{th} and 48^{th} hours significantly ($p<0.05$). However, while Xcelligence results was showing that caffeic acid decreased cell proliferation for all concentrations except 100 μM and 200 μM dosages during 24 hours, during 48 hours caffeic acid. Nevertheless 50 μM concentration revealed the same effect with control group in Xcelligence. When TUNEL assay which was applied with 50 μM dosage was considered, it was determined that caffeic acid had no effect on cell apoptosis. In this case Xcelligence and TUNEL assay confirmed each other.

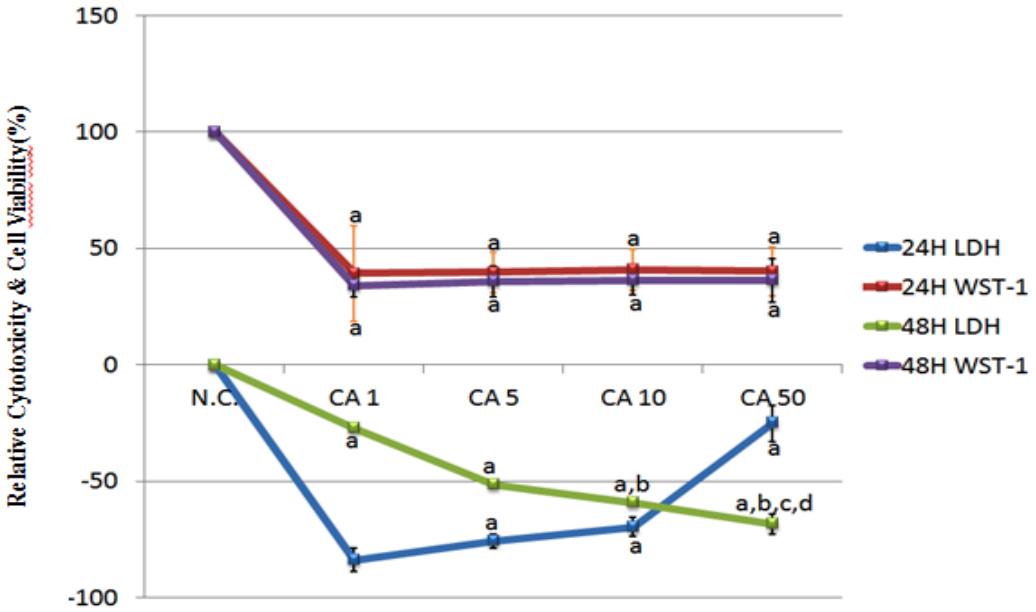

Figure 3.1 Results of the LDH and WST-1 assays treated for 24th and 48th hours. with different concentration of CA on HeLa cells.

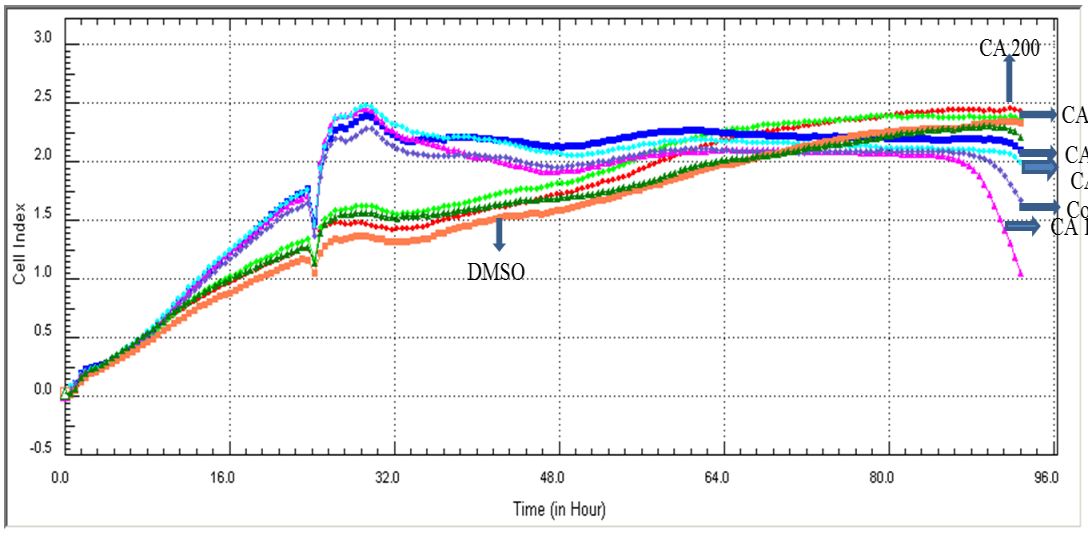

Figure 3.2 Results of the Xcelligence system treated for 24th and 48th hours with different concentration of CA on HeLa cells.

3.1.1 The Effects of Caffeoyl Malic Acid on Hela Cells

At the period of 24 and 48 hours in LDH assay caffeoyl malic acid had no effect on different concentrations. In WST-1 assay, caffeoyl malic acid did not affect cells at 24th hours. After Xcelligence assay it was observed that during 24th hours of 1, 5 and 10

µM caffeoyl malic acid did not influence cells but decreased cell number in higher concentration significantly ($p< 0.05$). Over 48th hours, WST-1 was effective and Xcelligence results also pointed out that caffeoyl malic acid influenced cells in all concentration exception with 200 µM dosage. Moreover, results of TUNEL assay 50 µM dosage of caffeoyl malic acid had apoptotic effect on cells after 48th hours. Therefore, results of Xcelligence, TUNEL and WST-1 assays confirmed each other.

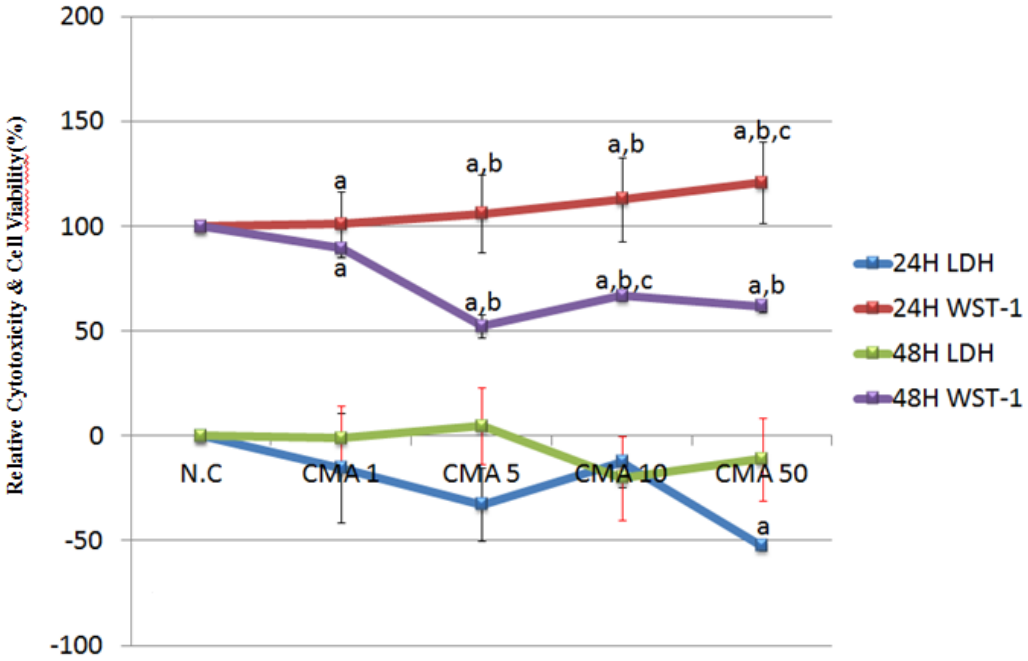

Figure 3.3 Results of the LDH and WST-1 assays treated for 24th and 48th hours with different concentration of CMA on HeLa cells.

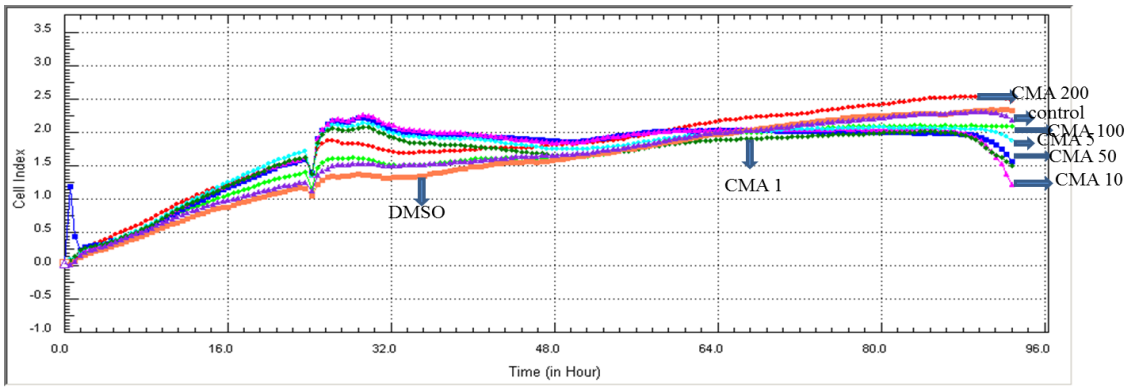

Figure 3.4 Results of the Xcelligence system treated for 24th and 48th hours with different concentration of CMA on HeLa cells.

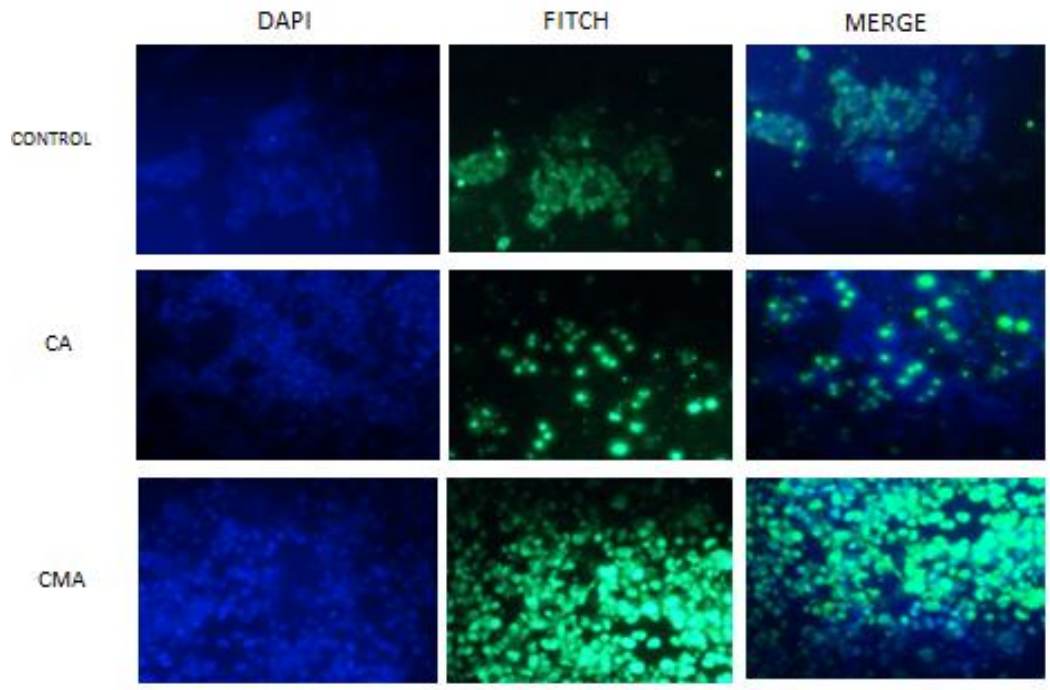

Figure 3.5 The results of TUNEL assay for positive control (Mitomycin C), CA(50 µM) and CMA (50 µM) on HeLa cells at 48th hour.

3.2 THE EFFECTS OF CAFFEIC ACID ON AGS CELLS

Caffeic acid which was applied on AGS cells gave rise to cell proliferation at 24^{th} and 48^{th} hours. In high concentrations, dependent on increased LDH, cell number decreased. But even so cell number was higher than cell number in control group. Xcelligence results were consistent with WST-1. So in Xcelligence result, except 200 µM dosage groups treated with caffeic acid had more cell numbers than control group. According to TUNEL results, caffeic acid had apoptotic effect on AGS cells in 50µM concentration at 48^{th} hours. In short, WST-1 and Xcelligence results confirm each other but TUNEL results don't.

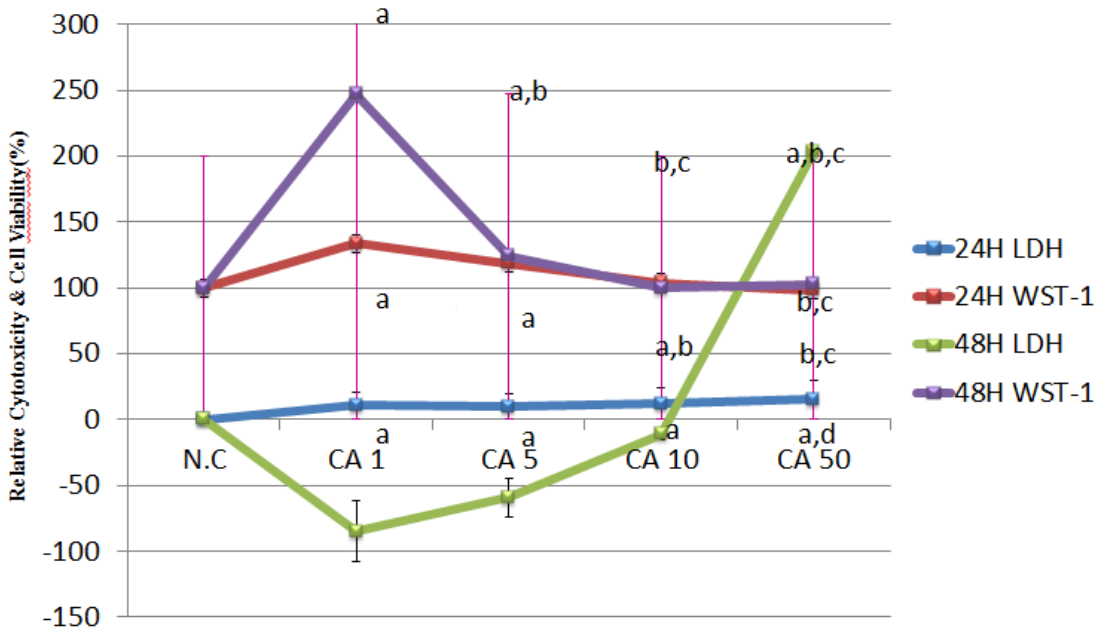

Figure 3.6 Results of the LDH and WST-1 assays treated for 24th and 48th hours with different concentration of CA on AGS cells.

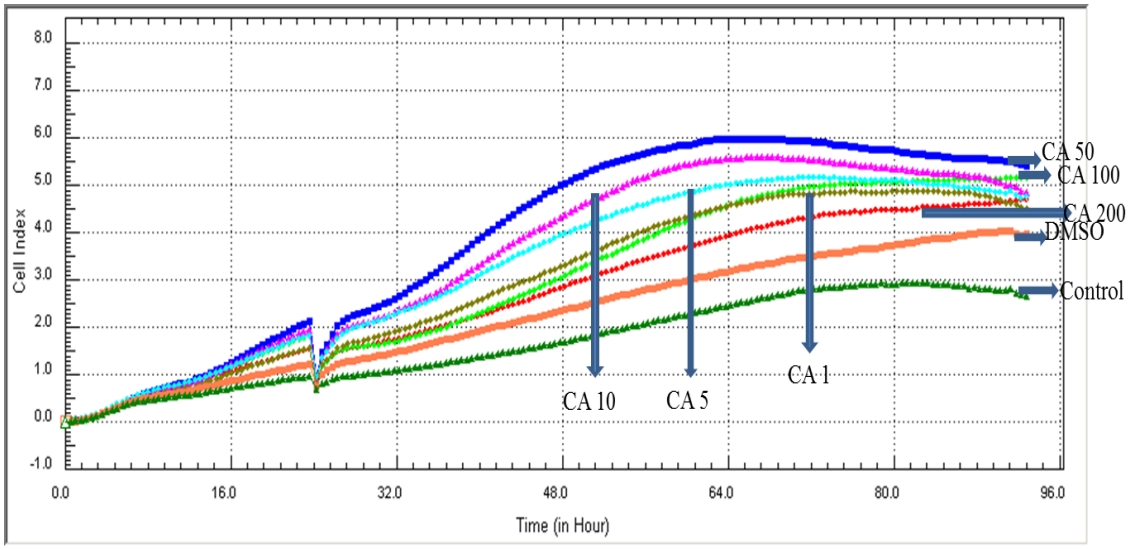

Figure 3.7 The results of Xcelligence treated for 24th and 48th hours with different concentration of CA on AGS cells.

3.2.1 The Effects Of Caffeoyl Malic Acid on AGS Cells

Caffeoyl malic acid did not affect AGS cells through both WST-1 and LDH assays at 24th hours. At 48th hours depending on cell number which were seen on

WST-1 data was decreased, quantity of LDH decreased, either. Xcelligence results marked that there was no significant change on AGS cells for all concentrations throughout both 24th and 48th hours. Also, TUNEL results displayed 50 µM dosage of caffeoyl malic acid had apoptotic effect on cells. As a result, except Xcelligence, WST-1 and TUNEL results confirmed each other.

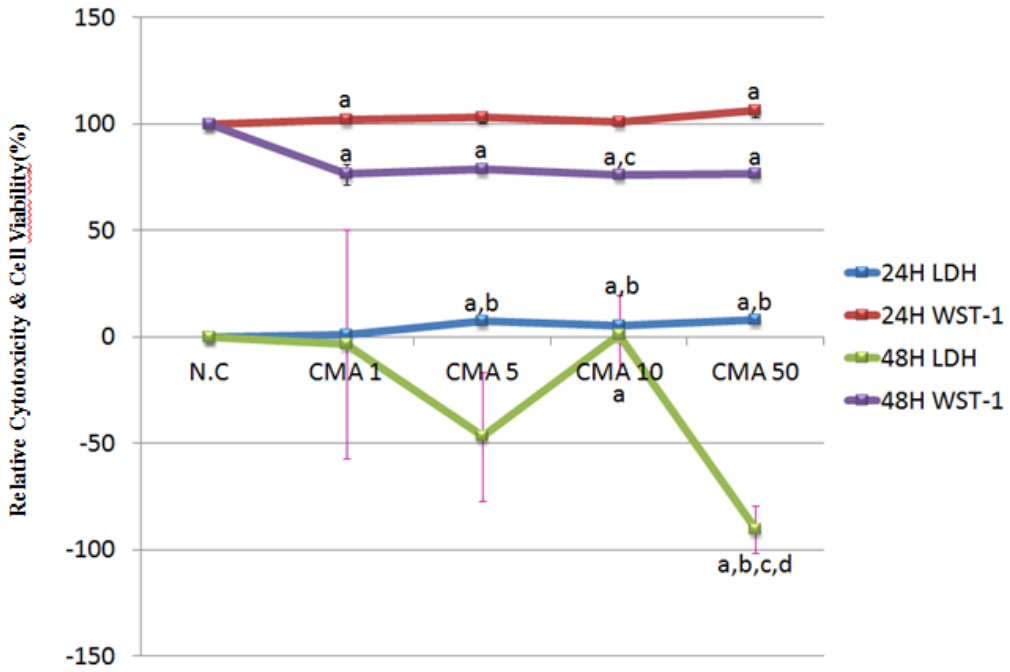

Figure 3.8 Results of the LDH and WST-1 assays treated for 24th and 48th hours with different concentration of CMA on AGS cells.

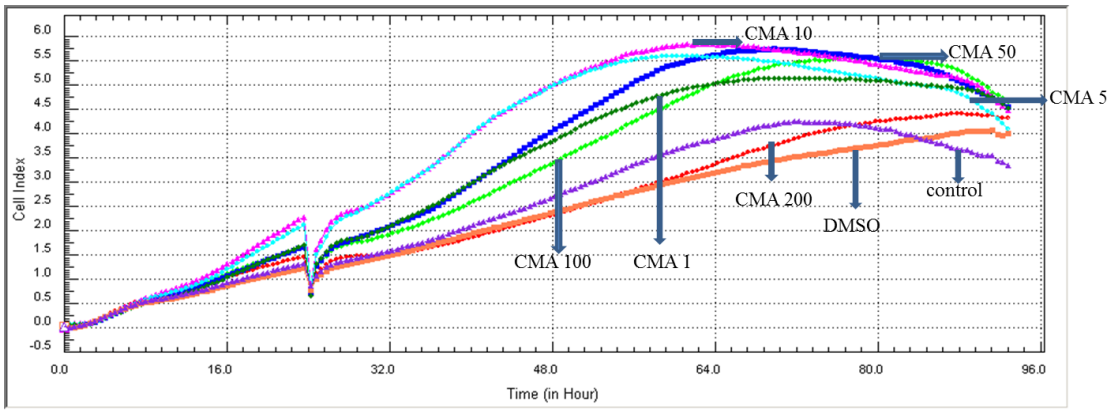

Figure 3.9 The results of Xcelligence treated for 24th and 48th hours with different concentration of CMA on AGS cells.

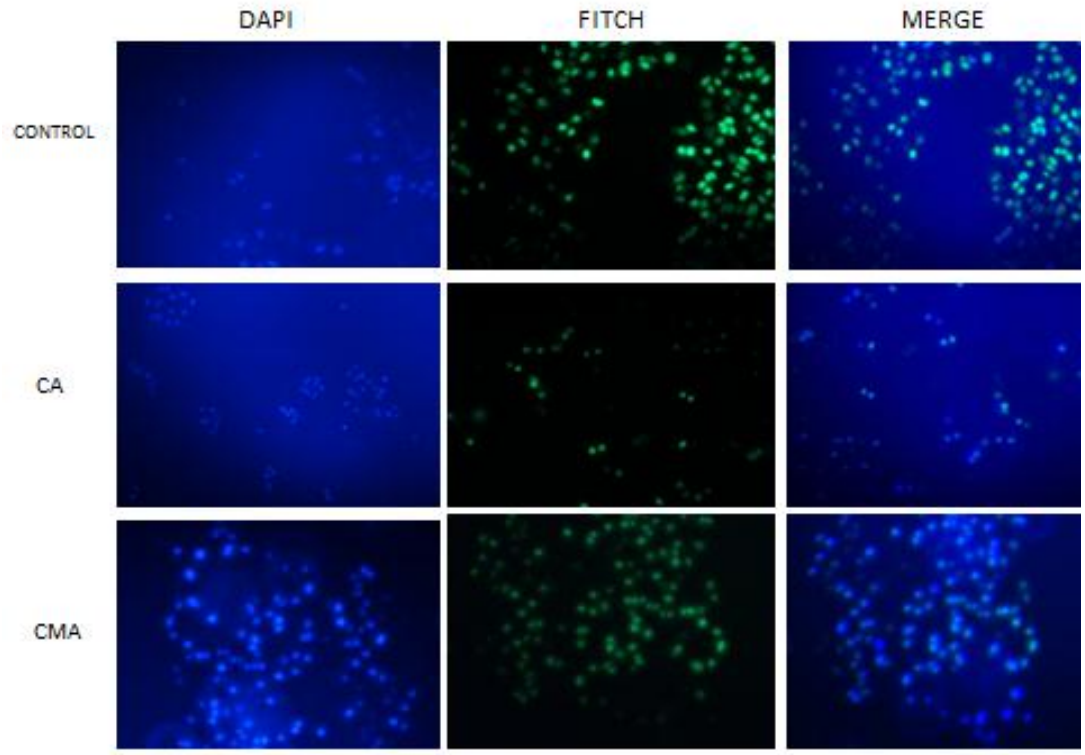

Figure 3.10 The results of TUNEL assay for control (Mitomycin C), CA(50 µM) and CMA (50 µM) on AGS cells.

3.3 THE EFFECTS OF CAFFEIC ACID ON HEK CELLS

According to WST-1 results, caffeic acid which was applied on HEK cells proliferated over 24th hours and increased cell proliferation in 48th hours except 50 µM concenration. Depending on increased and decreased in cell number, LDH amount increased and decreased, too. Xcelligence results stated cells in 5, 10 and 50 µM concentrations increased in number and dosages were not effective. In TUNEL results 50 µM of caffeic acid did not give rise to apoptosis on HEK cells at 48^{th} hours. To conclude, Xcelligence and TUNEL results confirmed each other. According to this result caffeic acid had no effect on HEK cells.

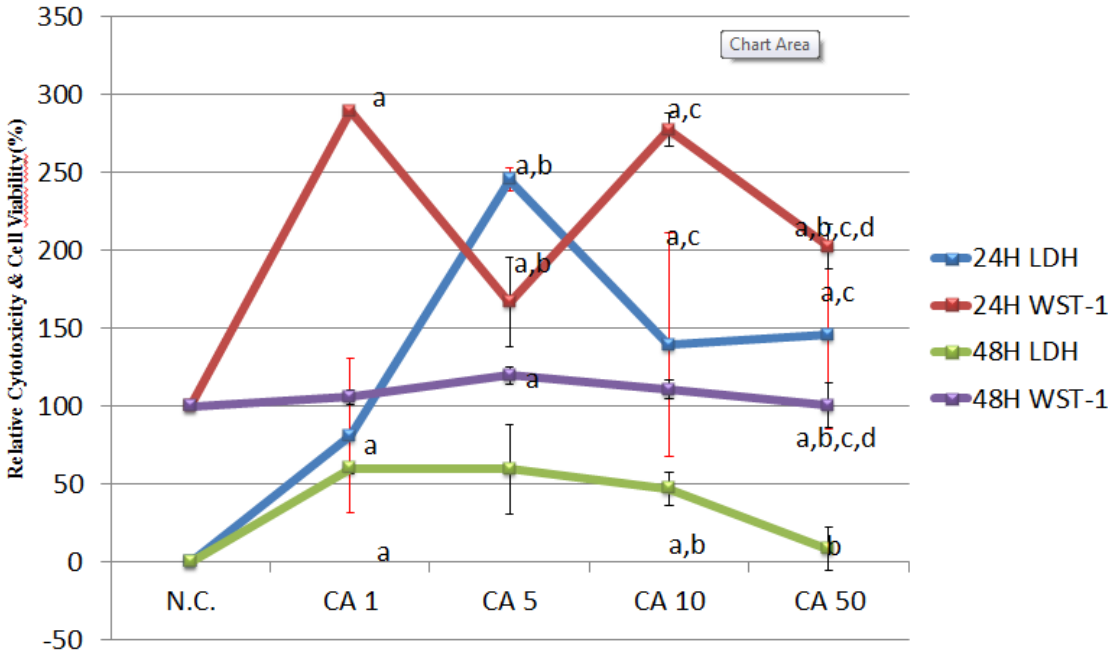

Figure 3.11 Results of the LDH and WST-1 assays treated for 24th and 48th hours with different concentration of CA on HEK cells.

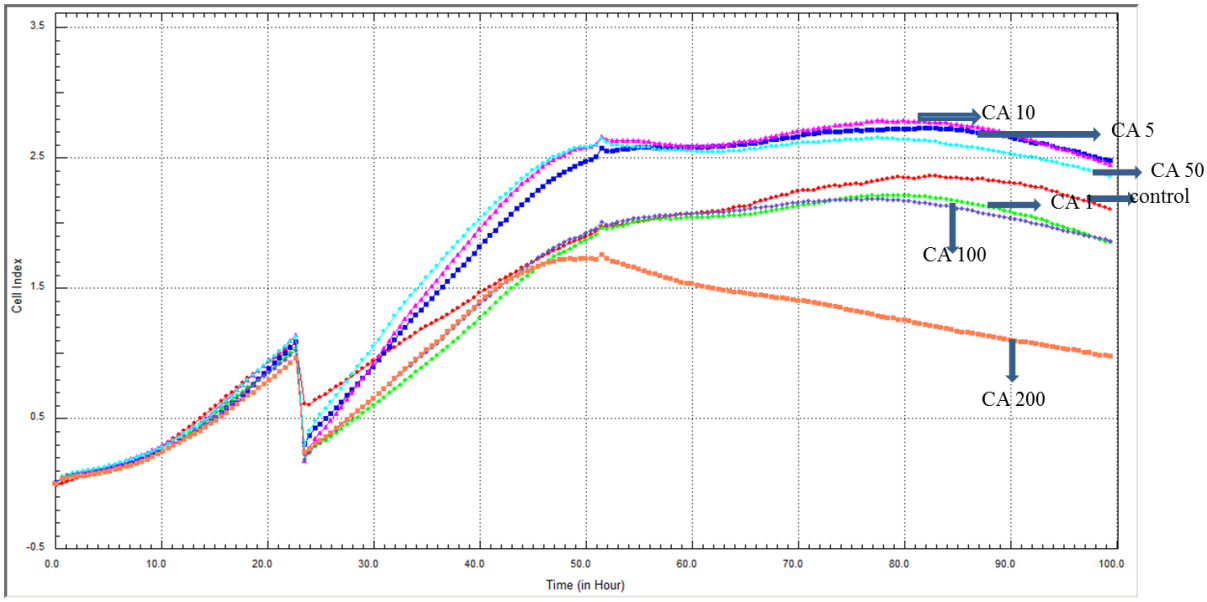

Figure 3.12 Results of the Xcelligence system treated for 24th and 48th hours with different concentration of CA on HEK cells.

3.3.1 The Effects of Caffeoyl malic acid on HEK Cells

It was observed that caffeoyl malic acid on HEK cells did not enhance LDH amount over 24th hours and did not change WST-1. Later Xcelligence results pointed out that caffeoyl malic acid is effective on cells, where 10 and 200 µM dosage is not included. After 48th hours quantity of LDH rised where WST-1 showed cell number decreased in high concentration. When 10 µM dosage is not included, results of Xcelligence assay reveal all concentrations of caffeoyl malic acid are effective. In another perspective, it has been tested in TUNEL assay in 48th hours, 50 µM of caffeoyl malic acid gave rise to apoptosis. Namely, TUNEL, WST-1 and Xcelligence results confirm each other. Ultimately, 50 µM of caffeoyl malic acid has effects on HEK cells over 48 hours.

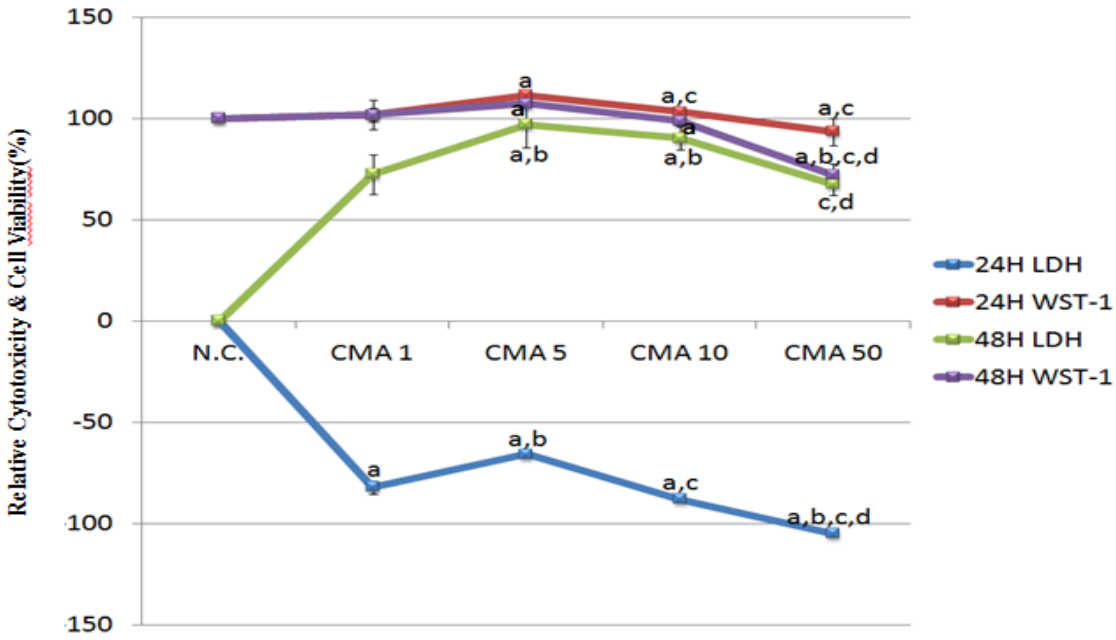

Figure 3.13 Results of the LDH and WST-1 assays treated for 24th and 48th hours with different concentration of CMA on HEK cells.

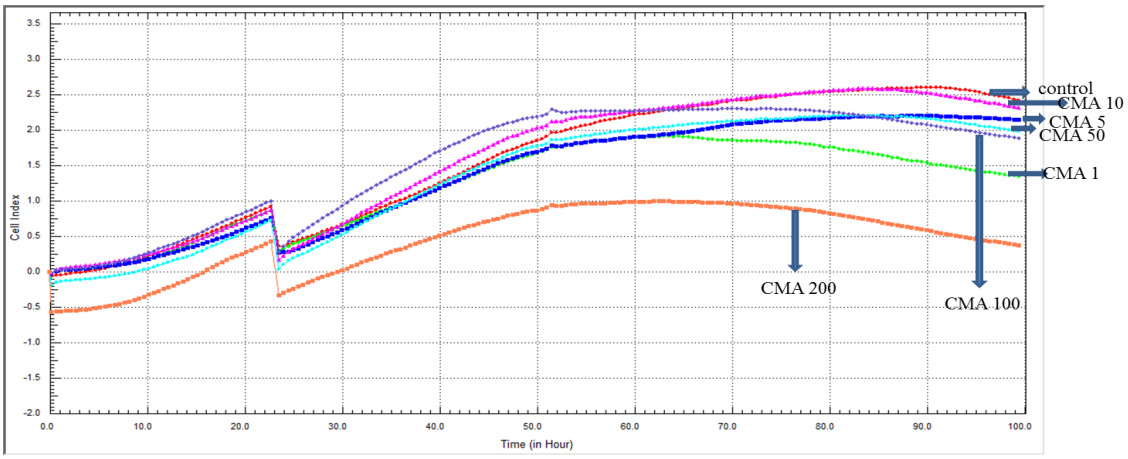

Figure 3.14 Results of the Xcelligence system treated for 24th and 48th hours with different concentration of CMA on HEK cells.

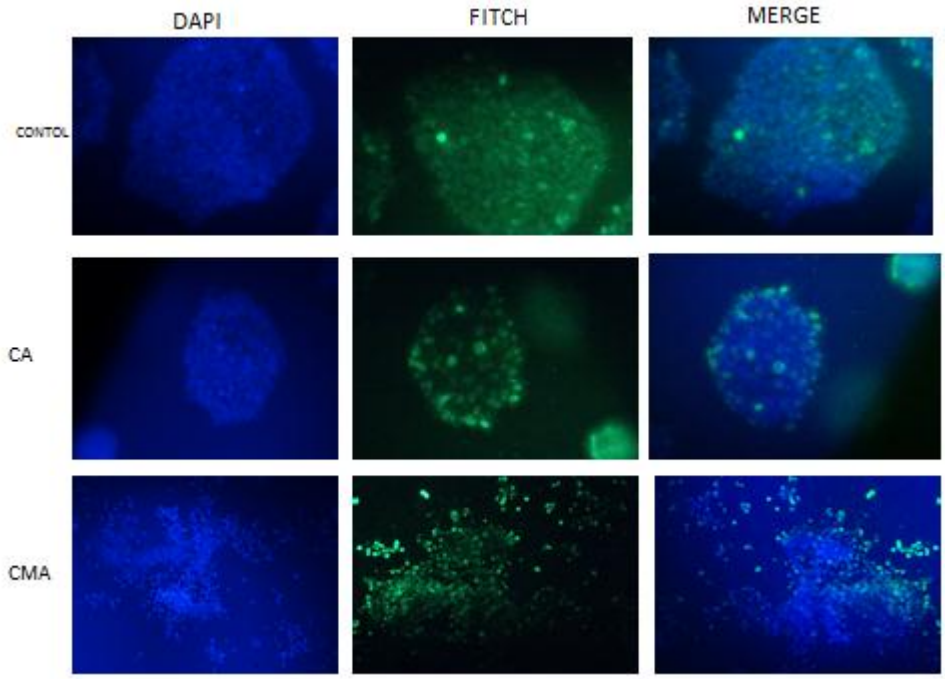

Figure 3.15 The results of TUNEL assay for control (Mitomycin C), CA(50 µM) and CMA (50 µM) on HEK cells.

3.4 THE EFFECTS OF CAFFEIC ACID ON U87 CELLS

Effects of caffeic acid on U87 cells were over 24th hours caffeic acid increased cell number in WST-1 assay significantly ($p<0.05$). This case showed a difference in Xcelligence results. According to Xcelligence, number of cells in all concentrations of

caffeic acid was less than WST-1. 48th hours for both WST-1 and Xcelligence assays, caffeic acid decreased cell number. At 24th hour related to increased in cell number, LDH amount also increased but over 48th hours depending on decreased in cell number, LDH volume decreased. On the other hand, in TUNEL results 50 µM dosage of caffeic acid induced apoptotic effect. At the end all results confirm each other.

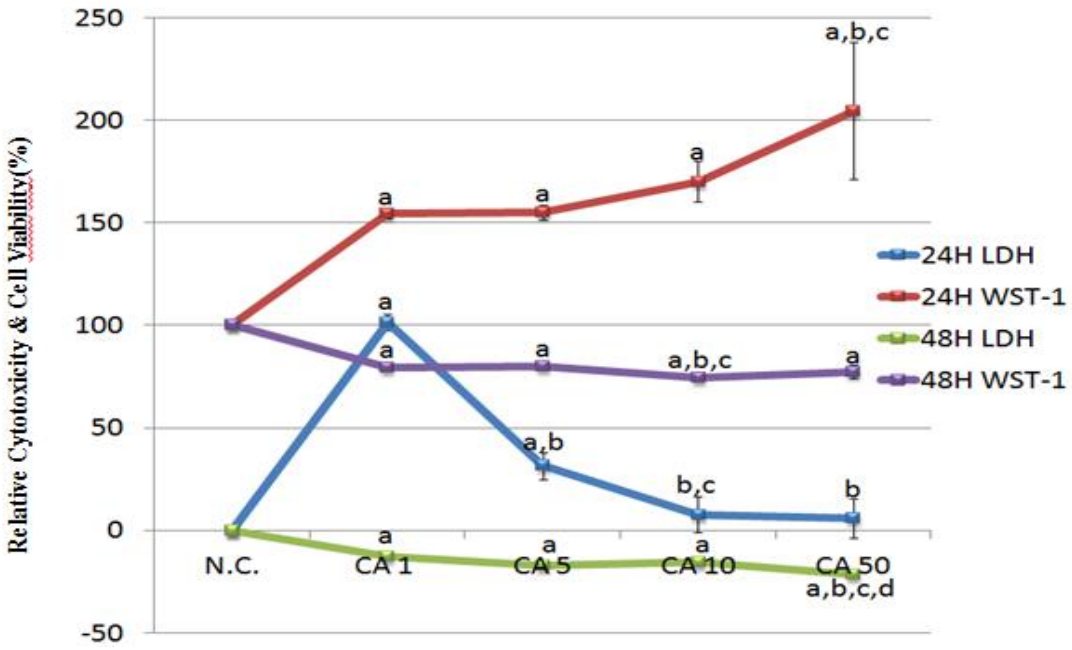

Figure 3.16 Results of the LDH and WST-1 assays treated for 24th and 48th hours with different concentration of CA on U87 cells.

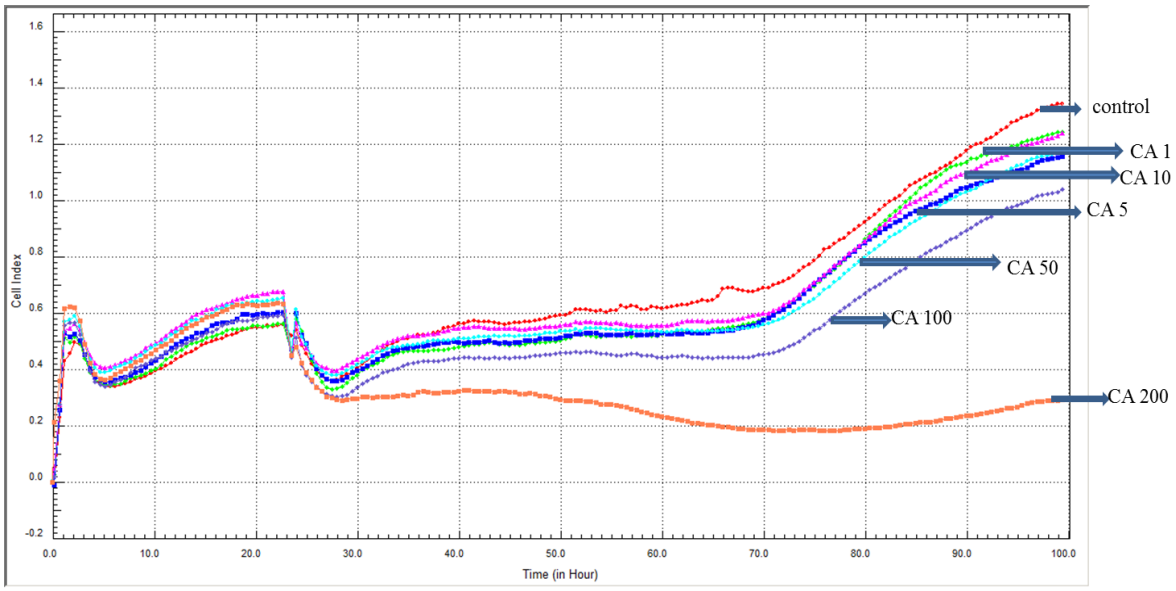

Figure 3.17 Results of the Xcelligence system treated for 24th and 48th hours with different concentration of CA on U87 cells.

3.4.1 The Effects of Caffeoyl Malic Acid on U87 Cells

LDH amount was not increased in the effect of caffeoyl malic acid on U87 cells. However, WST-1 result indicated that at both 24th and 48th hours of experiment cell number went down. Xcelligence result also revealed compatibility with WST-1 assay. Considering Xcelligence cell number for all concentration was below the level of control group. Caffeoyl malic acid for 50 µM concentration in TUNEL assay results along 48th hours stated that cells underwent apoptosis. All results endorse the other one. Last but not least 50 µM concentration of caffeoyl malic acid had distinct effect on U87 cells.

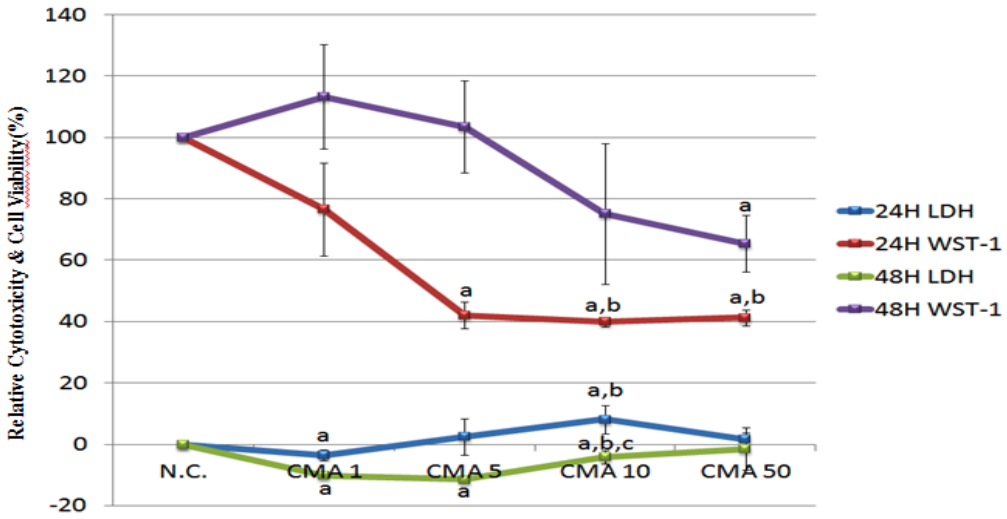

Figure 3.18 Results of the LDH and WST-1 assays treated for 24th and 48th hours with different concentration of CMA on U87 cells.

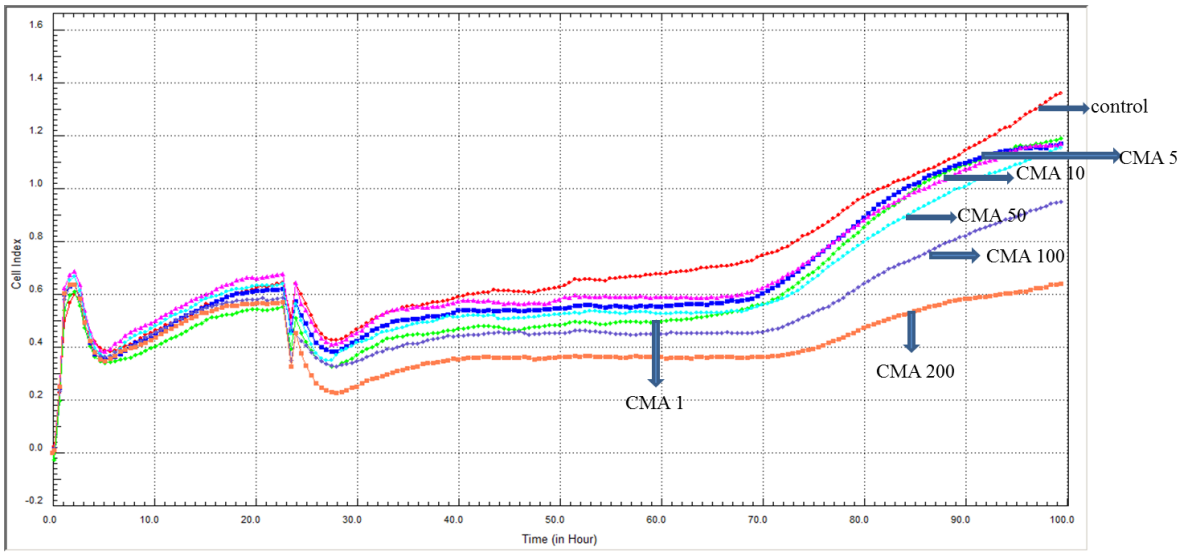

Figure 3.19 Results of the Xcelligence system treated for 24th and 48th hours with different concentration of CMA on U87 cells.

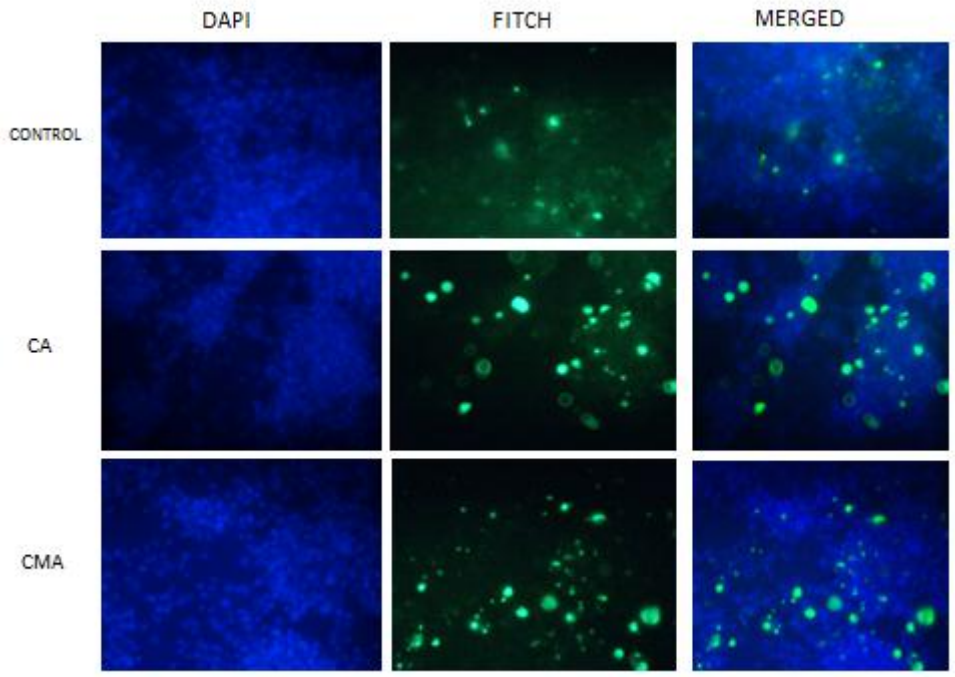

Figure 3.20 The results of TUNEL assay for control (Mitomycin C), CA(50 μM) and CMA (50 μM) on U87 cells.

3.5 TUNEL RESULTS

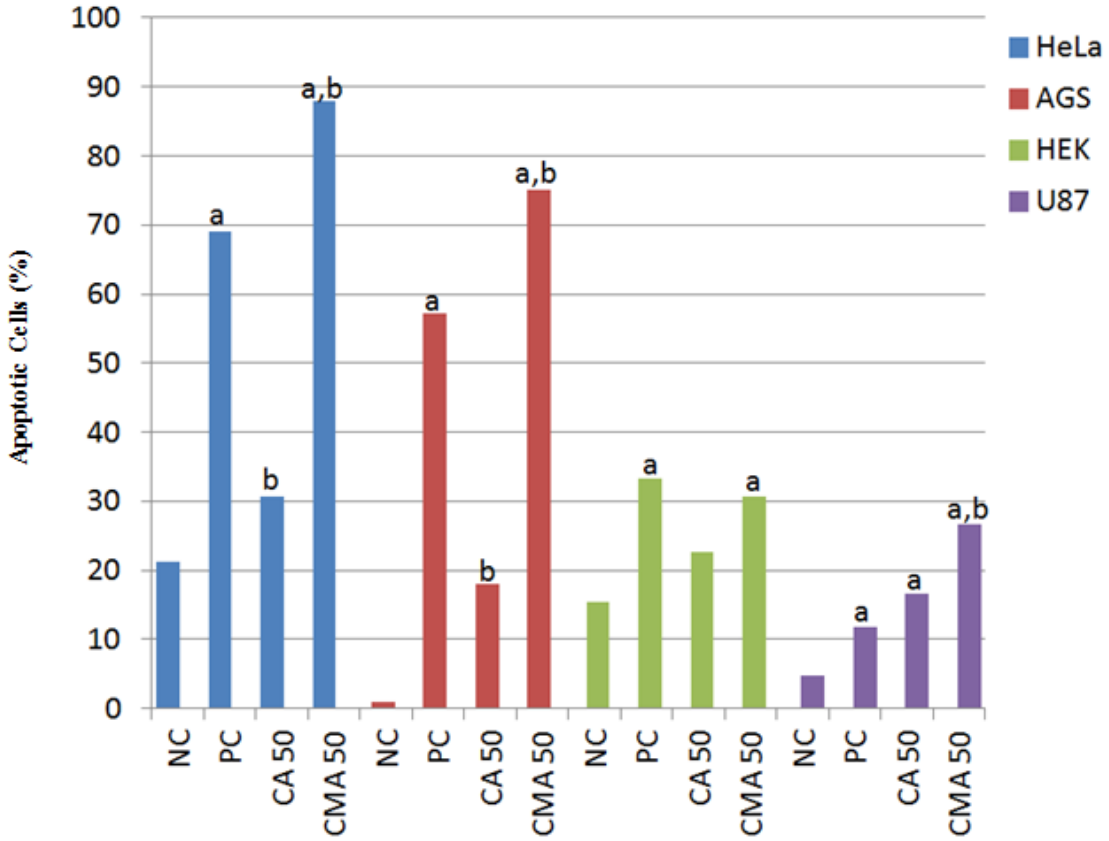

Figure 3.21 The results of TUNEL assay for CA(50 µM) and CMA(50 µM) on HeLa, AGS, HEK and U87 cells.

GROUPS	Values		DNA Fragmentation % Cells
HELA	**APOPTOTIC**	**NORMAL**	
POZİTİVE CONTROL	78	35	% 69,02
NEGATİVE CONTROL	35 e	130	% 21,21
CA 50	40 e	90	% 30,76
CMA 50	110 e, f	15	% 88
AGS			
POZİTİVE CONTROL	60 45		% 57,17
NEGATİVE CONTROL	0 150	a	% 0
CA 50	18 84	a,b	% 18
CMA 50	75 40	b	% 75
HEK			
POZİTİVE CONTROL	50	100	% 33,33
NEGATİVE CONTROL	20 110	c	% 15,38
CA 50	25	85	% 22,72
CMA 50	80 d	180	% 30,76
U87			
POZİTİVE CONTROL	78 35		% 11,76
NEGATİVE CONTROL	35 130	g	% 4,76
CA 50	40 90	h	% 16,66
CMA 50	110 15	g,h	% 26,66

Figure 3.22 AGS pa≤0.05 compared with p.control group; bp≤0.05 compared with n.control; HEK cp≤0.05 compared with p.control group; dp≤0.05 compared with n.control; HELA ep≤0.05 compared with p. control group; fp≤0.05 compared with n.control; U87 gp≤0.05 compared with p. control group; hp≤0.05 compared with n.control group; one way ANOVA test.

CHAPTER 4

DISCUSSIONS

Caffeic acid is a known plant phenolic acid, has been reported that caffeic acid acts as an antitumor promoter on forestomach, liver, skin carcinogenesis, oral cancer cell growth and suppress colon carcinogenesis. The effect mechanisms of caffeic acid were explained in previous studies. Caffeic acid inhibits NF-kB so slowing down the transcription of certain pro-inflammatory genes. NF-kB a eukaryotic transcription factor regulating the expression of gene such as COX-2, iNOS and 5-LO that are activated during inflammation by external agents such as mitogens or pro-inflammatory interleukins. Cyclooxygenase type 2 (COX-2) is responsible for the synthesis of prostaglandins (PGs). PGs play roles in cell proliferation, mitosis, apoptosis and immune response many types of cancers (breast, colon, and lung) (Alok C. Bharti, 2002).

In addition that the treatment of HepG2 cells with CA can suppress PMA (phorbol 12-myristate 13-acetate)-induced MMP-9 (Matrix metallopeptidase 9) expression by inhibiting the binding activity of NF-kB in hepatoma and inhibition of the in vitro invasion of PC3 cells was carried out using CA treatment (Weng and Yen 2012). The treatment of HeLa cells with caffeic acid leads to the disappearance of the anti-apoptotic Bcl-2 protein on the mitochondria and the release of cytochrome C into the cytosol. Also during the apoptotic process the p53 protein is expressed by effects of caffeic acid. Tumor suppressor protein p53 stimulates a wide network of biochemical signals in response to DNA damage. (Chang, Hsieh et al. 2010). According to another effect mechanism, CA down regulated MAPK proteins. Previous studies show us the MAPK pathways are deeply involved in signaling for various immune responses, including apoptosis and implicated in neuro inflammatory events. They detected the

down regulation of MAPK pathway related genes such as CA-treated U-87 MG cellsinvolved in of cell cycle, cell proliferation, oxidative phosphorylation, anti-apoptosis, insulin signaling pathway by them (Sohn, Ko et al. 2009).

CA has been studied on HeLa, HT 29 cells, HepG2 cells, HCT 15 colon cancer cells, mammary gland adenocarcinomas (MDA-MB-231), lymphoblastic leukemia (MOLT-3), non-neoplasic fibroblasts from human embryonic lung tissue (L-132 cell line), lung carcinoma (A549), non-small lung carcinoma (H1299). Some of them are Nada Oršoli et al. (2004) were observed the CA influence on the growth of HeLa cells *in vitro.* Proliferating HeLa cells were exposed to different concentrations of CA (6.25, 12.5, 25 μg/ml) for 24th, 48th, or 72th hours. Growth inhibition with CA on HeLa cells was dose-dependent (Orsolic, Knezevic et al. 2004). However Wei- Chang et al. (2010) were also studied the mechanism by which caffeic acid inhibits cell proliferation and the pathway of its pro-apoptotic effects are examined in HeLa. They treated cells with various concentrations of caffeic acid (0.5, 1, 2.5, 5 or 10mM) for 24 hours and showed that cell viability reduced from 58% after incubation with 1mM caffeic acid to 37% after treatment with 10mM caffeic acid while DNA fragmentation was seen in 83.94% of cells after exposure to 10mM caffeic acid for 48 hours. These findings indicated that caffeic acid induced apoptosis via the mitochondrial apoptotic pathway (Chang, Hsieh et al. 2010). Mirella et al. (2001) studied the cytotoxicity and apoptotic effects of caffeic acid in the human monocytic line U937. They observed the exposure of U937 cells to up to 100 μM CA had no cytotoxic effect. Following 48 h incubation with increasing levels of CA, DNA fragmentation was not detectable in U937 cells. They used in this study (50 and 100 μM), CA treatment is related with a significant inhibition of Cer-induced apoptosis (Mirella Nardini 2001). In a previous study, Jaganathan (2011) had observed inhibitory activity of caffeic acid against colon carcinoma cell line HCT 15. They used the different concentrations of caffeic acid for cell proliferation time dependent (100, 200, 300, 500, 600, 800, 1000 and 2500 μl). They determined inhibition of CA on cell number and increasing of apoptotic effects on treatment cells at high concentration.

On the basis of these previous studies, we decided to use the high concentration of CA that was 50 μM. We investigated cytotoxic, cell proliferation and apoptotic effects of CA on HeLa, AGS, HEK and U87 cell lines. Our study indicates that CA decreased cell proliferation of HeLa cell in dose dependent manner both 24^{th} and 48^{th} hours.

However CA had not cytotoxicity effects on HeLa cells. According to the Xcelligence result after 48th hour at all concentrations of CA except 50 μM reduced cell proliferation. Also WST-1 assay showed the cell proliferation was decreased at all concentrations of CA. We have studied on apoptotic effect of CA in HeLa cells. According to our TUNEL results CA has not apoptotic effect on HeLa cells. The inhibition of cell proliferation may be affected the necrotic effect of CA, these effects have not been studied in this thesis. Another cancel cell line was AGS cells. Although cell proliferation were increased along with 24 and 48 hours, in high concentration dependent on increased in LDH, cell number were decreased there were not apoptotic effects on AGS cells. Our data point out that HEK cells according to results, cell proliferation increased over 24 hours and in 48 hours in dose dependent. Cytotoxicity effects of CA on cells depending on increased and decreased in cell number. CA did not give rise to apoptosis on HEK cells depending on dose and time. Finally we investigated the cell proliferation and apoptotic effect of CA on U87 cells. Our study determined that cell proliferation reduced depending on time manner. Cytotoxicity effect of CA increased due to the increase in cell number and decreased depending on the reduction in cell number. We observed apoptotic effect of CA on U87 cell depending on time and dose manner. In the literature there is no any study about anti-proliferative effects of CA on HEK, AGS and U87 cell lines. Briefly, CA has anti-proliferative and apoptotic effects on HeLa and U87 cell lines. It didn't decrease cell proliferation and increase apoptosis in AGS and HEK cells.

Caffeoyl malic acid is another phenolic compound of *U. dioica*. But there are not any study about cell proliferation, cytotoxicity and apoptotic effect of CMA on cancer cell lines. So we investigated the CMA on AGS, HEK, HeLa, and U87 cell lines. Our studies show that CMA was applied on HeLa cells that result there were no cytotoxic effects but cell proliferation was decreased and apoptotic effects were observed depending on time and dose manner. The other cancer type was AGs cell lines. Cell proliferation reduced according to this result cytotoxicity also decreased dependent on dose and time manner. CMA did not affect AGS cells through Xcelligence result. However agreement of WST-1 result determined apoptotic effects of CMA depending on time and dose. In our Xcelligence result didn't confirm other experiments data. It may be experimental error. When we add the CMA on AGS cell line, control group's cell number was lower anyway. As you can see at Figure 3.9 from 42th hour to the end

of measurement the number of AGS was decreasing. Dose and time-dependent inhibition of CMA on HEK cells showed that cytotoxicity decreased depending on the reduced of cell number at high concentration. We observed apoptotic and anti-proliferative effects of 50μM CMA on HEK cells. Finally we studied the effects of CMA on U87 cells. This was dose-dependently associated with a decreased in cell proliferation at 48^{th} hour but there were not cytotoxic effects on U87 cells. However we demonstrated increasing accumulation of apoptotic cells after CMA treatment according to the dose and time. Our studies showed that CMA has anti-proliferative and apoptotic effects on HeLa, AGS, HEK and U87 cell lines especially at 50μM and 48^{th} hour.

CHAPTER 4

CONCLUSION

In conclusion, CA in different concentrations has anti-proliferative effects on HeLa and U87 cancer cell lines. Especially 50 μM concentration of CA is the most effective dose on U87 cell line. On the other hand high concentration of CA is more effective than 50 μM concentration of CA for the other cell lines. Although in literature there are some studies effects of CA on several cancer cell lines including HeLa, HT 29 cells, HepG2, HCT 15, MDA-MB-231, MOLT-3, L-132, A549, H1299. The impact of CA on the HEK, AGS and U87 cell lines are unknown. Also, there is no any information about the anti-carcinogenic effects of CMA on cancer cell lines. On the basis of our studies CMA has anti-proliferative and apoptotic effect on Hela, and U87 cell lines dependent dose and time manner.

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